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EDITED BY

SIMON FLEXNER, M.D.

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THE LYMPHOCYTE IN NATURAL AND INDUCED RESISTANCE TO TRANSPLANTED CANCER.

III. The Effect of X-Rays on Artificially Induced Immunity.*

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(Received for publication, April 11, 1918.)

In previous communications it has been shown (*a*) that the normal chick embryo lacks the ability to destroy heteroplastic tissue grafts;[1] (*b*) that this lack of resistance disappears between the 19th and 21st days of incubation, and it is significant that the spleen develops at about this time;[2] (*c*) that the lack of resistance, seen during the early days of the incubation period, is replaced by a degree of resistance comparable with that observed in the adult animal if the embryo is supplied with a small bit of adult lymphoid tissue;[3] (*d*) that the natural resistance of adult animals to heteroplastic tissue grafts can be destroyed by the x-rays in proper dosage;[4] and (*e*) that the disappearance of tissue grafts in resistant animals is associated with an accumulation of lymphocytes about them.[5]

It is known[6] that mice injected subcutaneously with homologous living tissue cells, after an interval of about 10 days, are potentially immune to tissues subsequently inoculated from the same species; namely, mouse cancers. This potential immunity can be readily destroyed by exposing the animals to suitable doses of the x-rays in the interval between the immunizing dose and the cancer inocula-

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* A preliminary announcement of these experiments was made by Murphy and Taylor before the American Association for Cancer Research, New York, April 5, 1917, and was published in abstract form in the proceedings of this meeting (*J. Cancer Research*, 1917, ii, 504). Since this report a paper has appeared by Mottram and Russ, repeating and confirming these observations (Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917–18, xc, 1).

This investigation was carried out by means of funds from the Rutherford Donation.

[1] Murphy, Jas. B., *J. Am. Med. Assn.*, 1912, lix, 874.

[2] Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

[3] Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 513.

[4] Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.

[5] For review of literature see Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1.

[6] Imperial Cancer Research Fund, *Brit. Med. J.*, 1906, ii, 209.

tion. This finding was interpreted as being due to interference with the lymphoid blood crisis which has been shown to follow the tumor inoculation[7] and which has been thought to influence the subsequent resistance.

It is established that the potential immunity to cancer resulting from an homologous tissue injection is of the nature of a non-specific reaction, the resistance produced being directed toward a great variety of cancers and sarcomas as well as toward homologous normal tissues. Injection with one of the transplantable mouse carcinomata renders the animal resistant to that tumor alone and thus the reaction becomes specific. It seemed possible that while the lymphocyte might be a potent factor in bringing about the potential immunity, which is non-specific, after the resistance becomes specifically directed against a particular tumor, this cell might no longer play a part in the maintenance of the immunity. To test this point the following experiments were carried out.

Method.

Mice were immunized by an injection of homologous defibrinated blood beneath the skin of the back. 10 days later a bit of tumor (Bashford Adenocarcinoma No. 63) was inoculated into the left groin of each animal. A number of non-immunized mice were inoculated at the same time with the tumor in order to control its virulence. After the animals had been observed for a period of 3 weeks, the immune animals were divided and one group was subjected to small repeated doses of x-rays, the other being set aside for controls. A week later both groups were reinoculated in the right groin with the same tumor strain, the virulence of the strain being determined by simultaneous inoculation into normal mice. The x-ray dosage used in these experiments was one which previous experiments[8] had shown to be adequate to destroy the major portion of the lymphoid tissue without appearing to affect the general health of the animal.

Experiment 1.—Forty-nine white mice of the same approximate age, size, and weight, obtained from one source, at the same time, were injected beneath the skin of the back with 0.2 cc. of defibrinated mouse blood. 10 days later there was

[7] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

[8] Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397. Taylor, H. D., and Murphy, Jas. B., *J. Exp. Med.*, 1917, xxv, 609.

inoculated into the left groin of each a bit of Bashford Adenocarcinoma No. 63. At the end of the 3 weeks interval following inoculation, forty animals, or 81.6 per cent, were found to be immune. Of ten normal animals inoculated simultaneously as controls, nine, or 90 per cent, developed tumors. It is evident, therefore, that the tumor is freely transplantable to normal animals and that the resistance present in those previously injected with mouse blood represents an induced immunity.

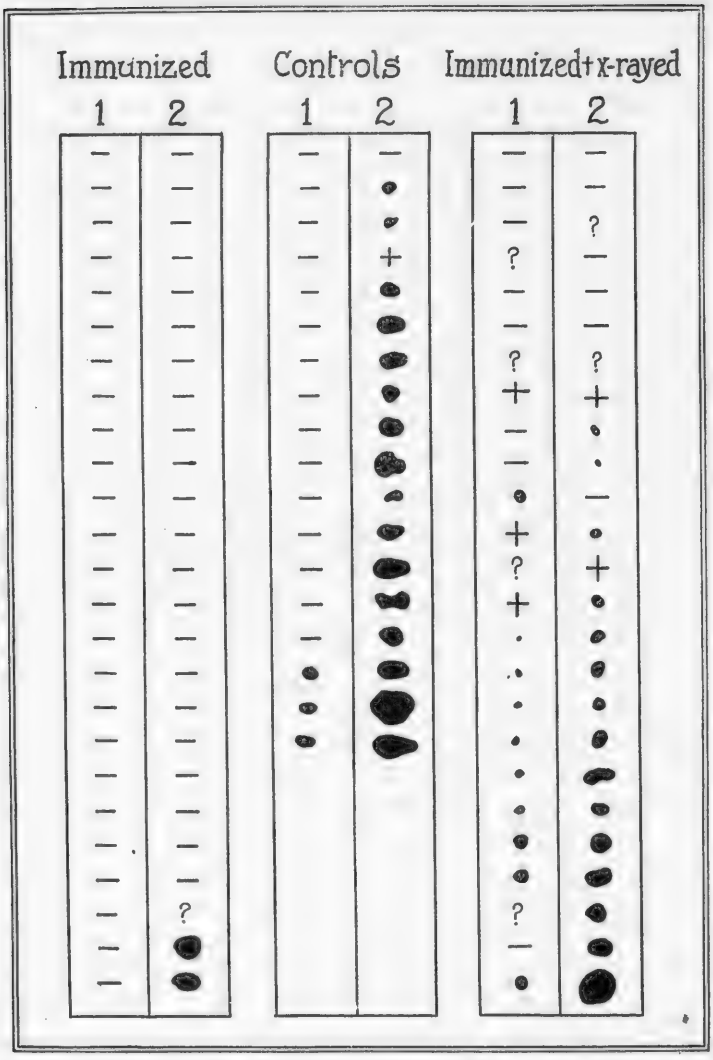
After a period of 3 weeks following the first inoculation with the tumor, the forty immune mice were divided into two groups. The first, comprising nineteen animals, remained untreated, while the second, comprising twenty-one animals, was exposed to the x-rays for 7 successive days. The dose of x-rays given at each exposure was as follows: a 2¼ inch spark-gap, 10 milliamperes, with a distance of 12 inches between the target of the Coolidge tube and the nearest point on the backs of the mice, time of exposure 2 minutes. After the seven exposures both groups of immune mice and eleven normal controls as well were inoculated with a later generation of the Bashford tumor in the right groin, with the results shown in Table I.

TABLE I.

Group.	Treatment.	Per cent of takes.
I	Immunized.	21.0
II	" and x-rayed.	52.4
III	Controls.	90.9

Of the animals artificially immunized against, inoculated with, and proven immune to a transplantable carcinoma, those which received no x-ray treatment before a second inoculation with the same tumor still showed a high degree of immunity, as only four of the nineteen, or 21 per cent, developed tumors. The second group of mice, which was x-rayed before the second tumor inoculation, showed a much smaller degree of immunity, eleven mice, or 52.4 per cent, yielding tumors. Of the controls, ten, or 90.9 per cent, developed tumors.

Experiment 2.—Fifty-two mice were immunized and inoculated with the mouse carcinoma in the manner indicated in Experiment 1; of these, fifty-one, or 98 per cent, proved to be immune. It happened that nine of the fifteen control mice developed tumors, so that 40 per cent showed natural immunity. During the 3rd week following the initial tumor inoculation twenty-five of the animals received seven daily x-ray treatments with a Coolidge tube, the following factors being used at each exposure: spark-gap 2¼ inches, milliamperes 10, distance from the target to the backs of the mice 12 inches, and the time of exposure 2 minutes.



TEXT-FIG. 1. Comparable sizes of the tumors in Experiment 2, as given in Table II. Column 1 shows the results 1 week and Column 2, 2 weeks after inoculation.

Twenty-five of the immune animals received no x-ray treatment. The two groups, fifty mice in all, together with eighteen control animals, were then inoculated in the right groin with a bit of a later generation of the Bashford tumor used in the primary inoculation. The results are shown in Text-fig. 1 and Table II.

TABLE II.

Group.	Treatment.	Per cent of takes.
I	Immunized.	12.0
II	" and x-rayed.	64.0
III	Controls.	94.4

Of the immune mice not treated with x-rays, only three, or 120. per cent, developed tumors. Of the immune animals x-rayed before the second inoculation, sixteen, or 64 per cent, developed tumors. There was tumor growth in seventeen, or 94.4 per cent, of the eighteen control animals.

TABLE III.

Group.	Treatment.	No. of mice.	Before x-ray treatment. Per cent of takes.	After x-ray treatment. Per cent of takes.
I	Immunized.	19*	10.5	73.3
II	Controls.	10†	90.0	90.0

* Fifteen surviving at second inoculation.

† Ten mice as controls for each inoculation of Group I.

Experiment 3.—As shown in Table III, nineteen mice were inoculated in the left groin with a bit of the Bashford tumor after having received, 10 days before the tumor inoculation, 0.2 cc. of defibrinated mouse blood. Two mice, or 10.5 per cent, developed tumors. Six doses of x-rays with the Coolidge tube were then administered on successive days, the daily dose depending on the following factors: spark-gap 2¼ inches, milliamperes 10, distance from target to mouse 12 inches, and time of exposure 1 minute. The fifteen mice which survived the period were then inoculated in the right groin with a later generation of the Bashford tumor and eleven, or 73.3 per cent, developed tumors. There was tumor growth in nine, or 90 per cent, of the ten animals used as controls to the first inoculation and the same percentage of growth in the same number of controls to the second inoculation.

Experiment 4.—Mice immunized and inoculated as indicated in the previous experiments were divided into two lots. After inoculation with the Bashford

tumor the first lot, consisting of nine mice, showed tumors in three, or 33.3 per cent. The second group of eight immune animals was given a series of x-ray treatments identical with those of Experiment 2, and 50 per cent developed tumors. There were 66.6 per cent of takes in the nine control animals (Table IV).

TABLE IV.

Group.	Treatment.	Per cent of takes.
I	Immunized.	33.3
II	" and x-rayed.	50.0
III	Controls.	66.6

Experiment 5.—Twenty-seven animals immunized as in the previous experiments were inoculated in the left groin with the Bashford tumor and to control further the immunity which was evident at this time were reinoculated with the same tumor. All proved to be immune after this rigid test. Seventeen of these immune mice gave 29.4 per cent of takes after a third inoculation with the same tumor to control the ten remaining ones which were also inoculated with this tumor after x-ray treatments on 3 successive days in the same manner as that employed in Experiments 1, 2, and 3, except that the time of exposure was 3 minutes for each of four exposures. Five of the second group, or 50 per cent, developed tumors. Four, or 40 per cent, of the ten control animals developed tumors (Table V).

TABLE V.

Group.	Treatment.	Per cent of takes.
I	Immunized.	29.4
II	" and x-rayed.	50.0
III	Controls.	40.0

Experiment 6.—Twenty mice were immunized and proved immune in the manner described in the preceding experiments, divided into two groups, and one group was given x-ray treatments with the Coolidge tube as follows: 2½ inch spark-gap, 10 milliamperes, 12 inch distance. Five of the daily exposures were of 2 minutes each and one for 5 minutes, the exposures being made during the 3rd week following the initial tumor inoculation. Of the seven animals so treated, 85.7 per cent developed tumors when given a second inoculation in the right groin with the Bashford tumor. A control group of thirteen untreated immune animals from the same lot of mice, inoculated at the same time, showed tumor growth in only 38.8 per cent. 60 per cent of the ten inoculated normal mice developed tumors. The results of the experiment are given in Table VI.

TABLE VI.

Group.	Treatment.	Per cent of takes.
I	Immunized.	38.8
II	" and x-rayed.	85.7
III	Controls.	60.0

Experiment 7.—Seven mice inoculated after being immunized in the usual way gave 28.2 per cent of takes. Inoculated a second time, only 14.2 per cent grew tumors. Of seven other mice, from the same group, 14.2 per cent developed tumors, after an inoculation with the Bashford tumor before treatment. After five daily exposures to the x-rays generated by a Coolidge tube there were 71.3 per cent of takes in this second group. The x-ray factors were the same as those used in Experiment 1. Control mice inoculated at the same time, with the same tumor, showed 71.3 per cent of tumors. The results of the experiment are given in Table VII.

TABLE VII.

Group.	Treatment.	First inoculation. Per cent of takes.	Second inoculation. Per cent of takes.
I	Immunized.	28.2	14.2
II	" and x-rayed.*	14.2*	71.3*
III	Controls.	71.3	—

* X-ray exposures after first and before second inoculation.

Experiment 8.—Twenty-eight mice were immunized, each with 0.2 cc. of defibrinated mouse blood, and divided into two groups. The first group of nine gave 11.1 per cent tumors after the first inoculation into the left groin and 37.5 per cent after the second inoculation into the right groin. They had no x-ray treatment. Of the second group of nine mice, x-rayed before the first inoculation, 55.5 per cent developed tumors. This group was again x-rayed and reinoculated into the right groin. 80 per cent developed tumors. A third group of immune animals was inoculated first in the left groin with the tumor, of which 20 per cent developed tumors. They were x-rayed, and upon reinoculation in the right groin 57.1 per cent of tumors resulted. The x-ray dosage was the same in both series of treatments given the second group of mice, as well as in the series of treatments which the third group received. The x-ray factors were identical with those of Experiment 1. The control mice gave 80 per cent of tumors. The results are given in Table VIII.

TABLE VIII.

Group.	Treatment.	First inoculation. Per cent of takes.	Second inoculation. Per cent of takes.
I	Immunized. No x-ray treatment.	11.1	37.5*
II	Immunized. X-rayed before first and second inoculations.	55.5	80.0†
III	Immunized. X-rayed before second inoculation.	20.0	57.1‡
IV	Controls.	80.0	—

* Only eight living at time of second inoculation.
† Only five living at time of second inoculation.
‡ Only seven living at time of second inoculation.

DISCUSSION.

The experiments described in this paper indicate anew that the lymphocytes are a potent factor in the immunity to cancer which has been studied in the mouse. Taken with other indications the evidence is growing in importance and conclusiveness of the part played by the lymphocyte in bringing about and in maintaining that condition. The main points of evidence now adducible are: (1) the accumulation of lymphocytes about a transplanted cancer graft in an immunized animal; (2) the rise in number in the circulating lymphocytes during the development of the immune state, irrespective of whether the type of immunity induced is artificial or natural; (3) the setting aside of the potential immunity by the x-rays where the dosage employed is sufficient to destroy a large part of the circulating lymphocytes; and finally, (4) as shown by the present experiments, the abolition of the potential immunity for a special tumor strain by means of the lymphocyte-destroying power of the x-rays. These specific points are further supported by the observations of Leo Loeb[9] on the part played by the lymphocyte in respect to homoplastic grafts of normal tissue, and by those of Murphy[3] on heteroplastic tissue grafts.

[9] Loeb, L., *J. Med. Research*, 1917, xxxvii, 229.

SUMMARY.

Mice artificially immunized against a transplantable carcinoma, inoculated, and proved immune, may be again rendered susceptible to the same tumor by exposure to the x-rays.

The immune animals which have not been treated with the x-rays preserve, to a large degree, their resistance to a second inoculation of the tumor in question.

THE PASSAGE OF NEUTRALIZING SUBSTANCE FROM THE BLOOD INTO THE CEREBROSPINAL FLUID IN ACTIVELY IMMUNIZED MONKEYS.

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(Received for publication, April 15, 1918.)

Previous experiments[1] have shown that in passively immunized monkeys neutralizing antibodies for the poliomyelitic virus can be made to pass from the blood into the cerebrospinal fluid by merely increasing the permeability of the meningeal-choroidal complex, through an aseptic inflammation induced by means of normal horse serum. The passive immunization is effected through the injection into normal monkeys of the blood serum of monkeys which have survived an attack of experimental poliomyelitis and which subsequently have had the immunity reinforced by subcutaneous injection of active virus (suspensions of spinal cord and brain of recently paralyzed monkeys preserved in glycerol). The order of the experiment was as follows: An aseptic meningitis was induced in normal monkeys by an intraspinal injection of 2 cc. of normal horse serum. The next morning, or about 16 hours later, about 10 cc. of the immune serum were injected intravenously. At intervals of 6, 9, and 24 hours fluid was withdrawn by lumbar puncture and employed for neutralization tests. The 6 and 9 hour specimens were combined so that the tests were actually made with samples of cerebrospinal fluid taken 6 and 9 hours and 24 hours after the horse serum was introduced. The control tests were made with normal horse serum. The procedure, as far as the actual neutralization is concerned, was identical with that of the present experiments. The results show that normal horse serum is devoid of neutralizing power for the virus; that at the expiration of 6 to 9 hours sufficient amount of the antibodies introduced into the blood had already passed into the cerebrospinal fluid to effect neutralization of the virus; and that the fluid withdrawn after 24 hours might no longer neutralize the virus perfectly.

These experiments were regarded as having a certain significance in respect to the specific therapy of poliomyelitis. Flexner and Lewis[2] and Flexner and Amoss[3] had already shown that the introduction of immune monkey or immune

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[1] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 499.

[2] Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 1780; lv, 662.

[3] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249; 1917, xxv, 525.

human serum into the meninges of monkeys prevented experimental infection with active poliomyelitic virus. Netter and his associates[4] first applied the principles of this observation to the treatment of cases of poliomyelitis in man. Later others, notably Amoss and Chesney,[5] reported series of cases treated in this manner with immune (convalescent) human serum. The promising results obtained led to the employment by others of normal sera. Thus Sophian[6] injected normal horse serum and Zingher[7] normal human serum intraspinally in cases of acute poliomyelitis. Their results were not definite, and yet they have been regarded as favorable in some instances.

Since normal serum exhibits no neutralizing action on the poliomyelitic virus *in vitro*, the possibility exists that the normal sera may serve merely to divert the neutralizing substances from the blood into the meninges by increasing the permeability of the meningeal-choroidal complex. It is now known that these bodies are detectable in the circulating blood of man in some instances as early as the 3rd day[8] of an attack of poliomyelitis. Hence the possibility exists that cases reacting favorably to intraspinal injections of horse serum may have endured long enough at the time of injection to be benefited by the diversion of immune bodies indicated.

Under these circumstances the diversion would take place not in a passively but in an actively immune person. It seemed desirable, therefore, to make actual tests upon actively immune monkeys. A number of animals which had recovered from attacks of experimental poliomyelitis were available. They had all subsequently been injected with virus suspensions to increase (reinforce) their immunity.

EXPERIMENTAL.

The specific experiments made to ascertain the presence of neutralizing substances in the cerebrospinal fluid were preceded by a series of

[4] Netter, A., Gendron, A., and Touraine, *Compt. rend. Soc. biol.*, 1911, lxx 625. Netter, A., *Bull. Acad. méd.*, 1915, lxxiv, series 3, 403. Netter, A., and Salanier, M., *Bull. et mém. Soc. méd. hôp. Paris*, 1916, xl, series 3, 299.

[5] Amoss, H. L., and Chesney, A. M., *J. Exp. Med.*, 1917, xxv, 581.

[6] Sophian, A., *J. Am. Med. Assn.*, 1916, lxvii, 426.

[7] Zingher, A., *Dept. Health, City of New York, Reprint Series, No. 54*, 1917.

[8] Kling, C., and Levaditi, C., *Études sur la poliomyélite aiguë épidémique*, Paris, 1913, 114.

tests to determine whether active complement is actually required to accomplish the destruction of the poliomyelitic virus *in vitro*. Diverse opinions on this point prevail.[9] Our previous experiments had led us to believe fresh complement not essential. On the other hand, irregularities not always readily explained sometimes arise in the course of the neutralization tests. As sometimes fresh and sometimes stored sera have been employed, the lack of uniformity has been attributed to the variation in the complement. As Experiment 1 indicates, inactive sera are perfectly neutralizing. The irregularities probably are to be accounted for rather by the quality of the virus, for a serum which contains in a given volume sufficient antibodies to neutralize a unit of virus of one degree of activity may fail to neutralize this unit of a more intense or active virus. Hence it is imperative to cover all tests on immunity in relation to poliomyelitis with adequate control observations. While the virus once adapted to monkeys by successive passages acquires and retains for a long time a marked virulence, yet quantitative fluctuations occur from time to time which are not predictable and heighten or depress the activity.

Experiment 1. Relation of Neutralization to Active Complement.—For this experiment a glycerolated virus (spinal cord and medulla) was employed. A 5 per cent suspension in isotonic saline solution was prepared, centrifuged, and filtered through a Berkefeld candle. *Macacus rhesus* A was inoculated intracerebrally with 2.6 cc. of a mixture of 2.4 cc. of fresh normal monkey serum and 0.2 cc. of virus filtrate. The mixture of filtrate and serum had been kept at 37°C. for 2 hours and in the refrigerator (4°C.) over night. 6 days after the inoculation the animal was ataxic, showed head tremor, and moved about slowly. Death took place on the 7th day. The autopsy showed the characteristic lesions of poliomyelitis. *Macacus rhesus* B received an intracerebral inoculation of the following: inactivated immune monkey serum 2 cc., inactivated normal serum 0.4 cc., virus filtrate 0.2 cc., mixed and treated as for *Macacus rhesus* A. No symptoms developed. *Macacus rhesus* C was a repetition of *Macacus rhesus* B in which active normal serum replaced the inactive. No symptoms appeared. For Monkey D an inactive human (convalescent) serum was employed in proportion of 2 cc. of serum to 0.2 cc. of virus filtrate. No symptoms developed.

This experiment yielded a clear and definite result. The neutralization of the virus is accomplished directly and without the inter-

[9] Landsteiner, K., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, viii, 449.

vention of fresh complement. This point has an interest in connection with the test with the cerebrospinal fluid which follows, since it shows that the presence of active complement can be disregarded.

Experiment 2. Passage of Neutralizing Substance into the Cerebrospinal Fluid.—This experiment was performed with cerebrospinal fluids withdrawn 6 and 9 hours respectively after the injection of 2 cc. of normal horse serum into the meninges of actively immune monkeys. The two fluids were combined and mixed with the virus filtrate in the manner of Experiment 1. The control tests were made with virus mixed with normal cerebrospinal fluid and with normal horse serum respectively. *Macacus rhesus* E (control) received an intracerebral inoculation of an incubated mixture of 1 cc. of normal cerebrospinal fluid, 0.3 cc. of isotonic saline solution, and 0.2 cc. of filtrate virus. On the 24th day ataxia, right facial paralysis, and weakness of extremities were present. The next day the extremities were paralyzed and death occurred. The autopsy showed lesions of poliomyelitis. *Macacus rhesus* F (control) received an intracerebral inoculation of an incubated mixture of 1 cc. of normal horse serum, 0.3 cc. of isotonic saline solution, and 0.2 cc. of filtrate virus. On the 6th day the extremities were paralyzed and a right facial paralysis existed. The animal was etherized and the autopsy showed marked lesions of poliomyelitis. *Macacus rhesus* G received an intracerebral inoculation of an incubated mixture of 0.2 cc. of virus filtrate and 1 cc. of the combined cerebrospinal fluid withdrawn from an actively immune monkey 6 and 9 hours respectively after the intraspinal injection of normal horse serum. 9 days later ataxia, right facial paralysis, tremor of head, and weak deltoids were present. The next day all the extremities were paralyzed and the animal was etherized. The autopsy showed lesions of poliomyelitis. *Macacus rhesus* H was an exact repetition of Monkey G. On the 12th day ataxia, paralysis of right arm, and weakness of many other muscles were noted. The paralysis extended somewhat and then gradually receded. Recovery with residual paralysis took place.

An immediate interpretation of this result would necessitate the conclusion that passage of neutralizing substances from the blood into the chemically inflamed meninges either did not take place in actively immune monkeys at all, or only inadequately in the period of 6 to 9 hours. Since in the passively immunized monkeys this period sufficed for the passage, some other explanation must be sought. Doubtless it is found in the discrepancy of the experimental procedure in the two series. In the case of the passively immunized monkey, the meningeal inflammation is induced about 16 hours before the immune serum is injected; hence a 9 hour specimen of cere-

brospinal fluid would be withdrawn about 25 hours after the horse serum was injected intraspinally. In the actively immune animals the fluid is withdrawn 9 hours after the horse serum is injected. In the one instance the inflammation is at its height, in the other in process of development when the cerebrospinal fluid is withdrawn. That this is the proper explanation is indicated by Experiment 3.

Experiment 3. Passage of Neutralizing Substance into the Cerebrospinal Fluid.— This experiment is a repetition and extension of Experiment 2. The manner of carrying it out was identical. Normal horse serum was injected intraspinally, and the periods at which the fluid was taken by lumbar puncture were 12, 24, and 48 hours. In the instance of the 48 hour withdrawal, a second injection of horse serum was made at the end of the first 24 hour period, in order to maintain the inflammation at a high level. Inoculations of monkeys with the cerebrospinal fluid and virus mixtures were made in duplicate, in order to cover any unforeseen variation in the results. *Macacus rhesus* I (control) received an intracerebral injection of an incubated mixture of 2 cc. of normal horse serum and 0.2 cc. of virus filtrate. On the 4th day the animal was excited; on the 5th, the extremities were all paralyzed and death resulted. The autopsy disclosed marked lesions of poliomyelitis. *Macacus rhesus* J and J′ each received an intracerebral inoculation of an incubated mixture of 0.2 cc. of virus filtrate and 1 cc. of cerebrospinal fluid withdrawn from an actively immune monkey 12 hours after an intraspinal injection of normal horse serum. No symptoms developed. *Macacus rhesus* K and K′ received similar injections of mixtures containing 24 hour specimens of cerebrospinal fluid and 0.2 cc. of virus filtrate. No symptoms developed. *Macacus rhesus* L and L′ received identical injections of mixtures containing 48 hour specimens of cerebrospinal fluid and 0.2 cc. of virus filtrate. No symptoms appeared.

This experiment is conclusive. It shows that beginning 12 hours after the normal horse serum is injected into the meninges of actively immune monkeys, and at a period when the inflammation induced may be regarded as marked, readily measurable quantities of the neutralizing antibodies were poured into the cerebrospinal fluid. This passage continues for 48 hours at least, that is considerably longer than in the passively immunized animals, as might have been predicted. Probably the passage would continue as long as the permeability of the meningeal-choroidal complex persisted. The results of this experiment indicate also that the explanation offered for the failure of Experiment 2 is probably the correct one.

The results of the experiments make clearer the manner in which recovery from poliomyelitis may be supposed to be brought about, and throw light on the probable value of a serum therapy. Immune bodies do not pass normally from the blood to the cerebrospinal fluid, which is, as it were, the lymph of the central nervous system (Mott[10]). In poliomyelitis, however, the entire vascular system of the meninges and affected portions of the solid nervous organs, as well as the structures of the choroid plexus, are often so severely injured as to be rendered readily permeable to the protein of the plasma and hence to immune bodies contained in it. The latter should therefore begin to appear in the cerebrospinal fluid just as soon as they begin to accumulate in the blood, and from that fluid permeate to the interior of the central nervous organs. From the moment this transfer of antibodies begins, the neutralization of the virus present in the nervous tissues would also begin; and gradually or quickly in the non-fatal cases an arrest of multiplication of the virus would be effected. That the cessation of the extension of the paralysis occurs very quickly in some cases is a matter of common observation. The presence of the neutralizing antibodies seems to be wholly determined by an excessive permeability of the blood vessels of the nervous system, for once their integrity is restored and the cerebrospinal fluid has returned approximately to normal composition, neutralizing antibodies can no longer be detected there.[11] By the time the acute process is at an end, the infection has run its course. There remains to be accomplished merely the restoration, as far as may be, of the organic and functional integrity of the injured structures.

CONCLUSIONS.

For the neutralization of the virus of poliomyelitis by antibodies, active complement is not required.

In carrying out immunity tests it is imperative to choose a virus of established grade of virulence and to make adequate control observations.

[10] Mott, F. W., *Lancet*, 1910, ii, 79.

[11] Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 205.

The neutralizing substances pass from the blood of actively immune monkeys into the cerebrospinal fluid when the permeability of the meningeal-choroidal complex is increased by an aseptic inflammation such as that induced by an intraspinal injection of horse serum.

The immunity bodies in effective neutralizing quantities can be detected in the cerebrospinal fluid as early as 12 hours and as late as 48 hours after the intraspinal injection of horse serum. Doubtless the passage continues as long as the inflammation persists.

This ability of the neutralizing substances to pass from the blood into the cerebrospinal fluid under conditions of inflammation doubtless plays an important part in arresting the multiplication of the virus on which the cessation and restoration of the poliomyelitic processes depend. The widespread involvement in the inflammatory conditions of the meninges, choroid plexus, and substance of the nervous organs, accompanied by severe lesions of the blood vessels in the last structures especially, opens the way widely for the passage of antibodies into the cerebrospinal fluid, whence all parts of the nervous tissues are reached, and also, probably, for direct transudation into the affected parts of the spinal cord and brain. The neutralization of the virus on which the continuance of the active pathological process depends is thus readily accomplished.

Under these circumstances the use of an alien specific immune serum to anticipate the action of the individual's own immunity products appears logical, while the employment of normal serum has no basis in experiment and would seem not to offer any therapeutic advantage whatever.

RESULTS OF PROPHYLACTIC INOCULATION AGAINST PNEUMOCOCCUS IN 12,519 MEN.

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The success of prophylactic vaccination against typhoid fever as demonstrated by the results obtained in the United States Army naturally suggests the effort to combat other prevalent and serious diseases by the same method. Among such diseases pneumonia easily ranks first. During the past winter it has been responsible for probably 80 per cent of the deaths in the various training camps in this country, and though the streptococcus has been the causative agent in many of these cases the pneumococcus has also played a prominent part.

Animal experiments have shown that it is easy to produce active immunity to pneumococci by the injection of small doses of dead organisms even in animals as susceptible as the mouse and rabbit, and this immunity persists for a considerable time. It is theoretically possible, therefore, to immunize man to the fixed types of pneumococci by injection of dead cultures.

The only place to our knowledge where preventive inoculation against pneumonia has been hitherto attempted has been among the workers in the mines in South Africa, where the disease occurs with great frequency and causes the death of large numbers of native workmen. The first inoculations on a large scale were carried out by Sir Almroth Wright (1) in 1911 and 1912. However, in these experiments no attention was paid to differences in types of pneumococci and this fact renders the interpretation of the results of the inoculations extremely difficult. In 1913 Dochez and Gillespie (2) published a classification of pneumococci and Lister (3) independently reported shortly afterwards a similar classification

of the pneumococci encountered in South Africa. Lister then undertook an experimental study of prophylactic inoculation against the various types of pneumococci in animals and man. He demonstrated that immunity can be produced in man against at least certain ones of these types either by subcutaneous or intravenous injection, more readily by the latter. He found that subcutaneous inoculation of 40 billion cocci of the strains he employed caused little if any toxic reaction in the guinea pig, rabbit, or man, and intravenous inoculation of 20 billion in the rabbit and 40 billion in man gave rise to but slight toxic reaction. On the basis of these experiments Lister undertook the prophylactic inoculation of large groups of miners against pneumococcus and has recently reported the results of his experiments. He at first advocated inoculation at 7 day intervals, each dose to consist of 6 billion cocci of each type against which immunity was desired. Subsequently he greatly reduced this dosage and gave three subcutaneous inoculations at 7 day intervals, each injection consisting of 2 billion of each type.

The workers in three different mines, the Crown, Premier Diamond, and De Beers Diamond, were inoculated with a vaccine composed of the three types of pneumococcus which were most prevalent in these mines. They were known as Types A, B, and C. Types B and C correspond to Types II and I, respectively, in Dochez and Gillespie's classification. Type A has not been encountered in America. In the De Beers Diamond Mine a fourth group was added, called Type H. In the De Beers experiment 1 billion of Type H was added to each injection, making a total dosage at each injection of 7 billion. The vaccinated miners were then observed over a period of 6 to 12 months and in all three mines a definite decrease in the incidence and mortality rate from pneumonia was observed. In the case of the Crown Mines, every case of pneumonia which occurred among the vaccinated individuals was studied bacteriologically and the type of pneumococcus determined. No cases of the types against which the men had been vaccinated (Types A, B, and C) developed during the 9 months of observation. Lister contends that this fact, namely the alteration of a relative group prevalence by means of specific group inoculation, is a more critical test of the efficacy of pneumonia prophylaxis than the simultaneous comparison of pneumonia rates in inoculated and uninoculated (control) groups when the comparison is based upon the erroneous assumption that all cases of disease due to the pneumococcus are bacteriologically indistinguishable. He emphasizes the probability that the protection of a considerable part of the community by inoculation lessens the number of carriers, and perhaps the virulence of the strains found in the community, and hence confers a definite benefit upon the uninoculated group which would affect the use of this group as controls in a statistical sense. Lister reported no unpleasant effects from the vaccine.

Bacteriological study of the first hundred cases of pneumonia occurring at Camp Upton showed that about 70 per cent were due to the pneumococcus and of these, about 50 per cent were caused by Type

I, II, or III. In consideration of this fact and of the results achieved by Lister in South Africa, it seemed desirable to employ prophylactic vaccination against the pneumococcus at Camp Upton. At the outset it was decided to incorporate only Types I, II, and III in the vaccine. Vaccination against Type IV was obviously not feasible on account of its many varieties. The preliminary problems to be determined were: First, how large a dose of such a vaccine could be administered without producing a severe reaction? Second, would the dose, injected three or four times subcutaneously, produce an efficient and demonstrable immunity?

Preliminary Experiments.

For the preparation of the vaccine preliminary experiments showed that agar media were unsatisfactory because of the small yield of organisms. Experiments in immunizing rabbits with pneumococci grown on a variety of media indicated that those obtained from 0.5 per cent glucose broth were as effective antigenically as those obtained from agar media or from plain broth, and as the yield from glucose broth is much more abundant this medium was adopted. To prevent autolysis, incubation was stopped after 12 to 14 hours. Centrifuging was carried out by the use of a continuous feed type of centrifuge (Sharpless laboratory centrifuge) which will take 8 to 12 liters of broth per hour and give a clearer supernatant fluid than is usually obtained from the bucket type of centrifuge. The cultures were killed before centrifuging by heating to 53°C. for $\frac{1}{2}$ hour. This conserves the antigenic effects of the culture. After centrifuging, the organisms were suspended in normal saline solution, shaken to secure even distribution, standardized by dilution and comparison of the opacity with known suspensions of pneumococci, and again heated at 55°C. for $\frac{1}{2}$ hour. Sterility of the vaccine was established by aerobic and anaerobic cultures and by subcutaneous inoculation into guinea pigs and intraperitoneal inoculation into mice. Tricresol was added to a concentration of 0.3 per cent as a preservative.

In order to determine the optimum dosage and interval of injection inoculations were given to forty-two adult volunteers in varying dosage and at different intervals, and the effect was gauged by means of tests made upon the serum taken 8 days after the last in-

jection. The character of the local and general reactions to the inoculation was also observed. The tests applied to the serum were the agglutination titer against the three types included in the vaccine and the protective power of the sera against these types injected into mice.

The agglutination titer was carried out with 24 hour plain broth cultures of the same strains used in preparing the vaccine. The serum dilutions used were 1:1, 1:3, 1:10, and occasionally 1:30. The tubes were incubated for 2 hours at 37°C. in a water bath, given a first reading, placed in the ice box over night, and then given the final reading. The results charted are based on the final reading.

For the protection experiments the method described by Dochez (4) was employed. A plain broth passage culture from the heart's blood of a mouse was used for each of the three strains employed in preparing the vaccine. Several dilutions of these three cultures were made with sterile broth so that 0.5 cc. of the dilutions would contain respectively 0.01, 0.001, 0.0001, 0.00001, and 0.000001 cc. of original culture. 2 parts of each serum were diluted with 3 parts of normal salt solution so that 0.5 cc. of the diluted serum contained 0.2 cc. of serum. With each serum mice were injected intraperitoneally with some or all of the following mixtures of diluted serum and diluted cultures.

Mouse 1, 0.5 cc. of diluted culture (equals 0.01 cc. of culture of Type I) + 0.5 cc. of diluted serum.

Mouse 2, 0.5 cc. of diluted culture (equals 0.001 cc. of culture of Type I) + 0.5 cc. of diluted serum.

Mouse 3, 0.5 cc. of diluted culture (equals 0.0001 cc. of culture of Type I) + 0.5 cc. of diluted serum.

Mouse 4, 0.5 cc. of diluted culture (equals 0.01 cc. of culture of Type II) + 0.5 cc. of diluted serum.

Mouse 5, 0.5 cc. of diluted culture (equals 0.001 cc. of culture of Type II) + 0.5 cc. of diluted serum.

Mouse 6, 0.5 cc. of diluted culture (equals 0.0001 cc. of culture of Type II) + 0.5 cc. of diluted serum.

Mouse 7, 0.5 cc. of diluted culture (equals 0.01 cc. of culture of Type III) + 0.5 cc. of diluted serum.

Mouse 8, 0.5 cc. of diluted culture (equals 0.001 cc. of culture of Type III) + 0.5 cc. of diluted serum.

Mouse 9, 0.5 cc. of diluted culture (equals 0.0001 cc. of culture of Type III) + 0.5 cc. of diluted serum.

As controls six mice were injected intraperitoneally as follows:

Mouse 1, 0.5 cc. of diluted culture (equals 0.00001 cc. of culture of Type I) + 0.5 cc. of normal saline solution.
Mouse 2, 0.5 cc. of diluted culture (equals 0.000001 cc. of culture of Type I) + 0.5 cc. of normal saline solution.
Mouse 3, 0.5 cc. of diluted culture (equals 0.00001 cc. of culture of Type II) + 0.5 cc. of normal saline solution.
Mouse 4, 0.5 cc. of diluted culture (equals 0.000001 cc. of culture of Type II) + 0.5 cc. of normal saline solution.
Mouse 5, 0.5 cc. of diluted culture (equals 0.00001 cc. of culture of Type III) + 0.5 cc. of normal saline solution.
Mouse 6, 0.5 cc. of diluted culture (equals 0.000001 cc. of culture of Type III) + 0.5 cc. of normal saline solution.

The time of injection of each mouse was noted and the number of hours to time of death recorded. When a mouse had survived over 140 hours it was recorded as "survived." Upon dying the mice were autopsied and films made from the peritoneal exudate and stained to determine the presence of Gram-positive diplococci. From the control mice, upon dying, cultures were made from the heart's blood and the type was verified by agglutination. The range of survival exhibited in the controls throughout the tests is shown in Table I.

TABLE I.

Mouse Controls for Protection Tests.

	Type I.		Type II.		Type III.	
	0.00001 cc.	0.000001 cc.	0.00001 cc.	0.000001 cc.	0.00001 cc.	0.000001 cc.
Minimum.....	28*	31	18	16	14	33
Maximum.....	36	36	40	32	36	45

*The figures indicate the hours of survival of each mouse after injection.

All inoculations except in Individual 9 were given subcutaneously. The first one inoculated, Individual 9, received two subcutaneous inoculations of 16 billion cocci of each type at each inoculation, 4 days apart, followed 4 days later by one-tenth this dose intravenously. An extensive area of redness with some tenderness followed the subcutaneous inoculations, reaching the maximum in 48 hours but without incapacitating the subject; there was no accompanying general

reaction. The intravenous injection was followed 48 hours later by a slight malaise and a temperature of 100.4°F. of a few hours' duration.

All other inoculations were given subcutaneously as this was considered the only method feasible for use later on a large scale in the camp.

Individuals 1 to 8 (Table II) received a first dose subcutaneously of 8 billion cocci of each type. In four adults a moderate local reaction resulted. In two, however, the local reaction was severe, leading

TABLE II.

Individuals Receiving a Single Large Inoculation.

Individual No.	Total dosage in billions.			Reaction.		Tests on sera 7 days after injection.																
						Agglutination.									Mouse protection.							
						Type I.			Type II.			Type III.			Type I.			Type II.			Type III.		
	Type I.	Type II.	Type III.	Local.	General.	1:1	1:10	1:30	1:1	1:10	1:30	1:1	1:10		0.01 cc.	0.001 cc.	0.0001 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.01 cc.	0.001 cc.	0.0001 cc.
1	8	8	8	Sl.*	N.	++	−	−	++	−	−	−	−		S.	S.	S.	27	S.	S.	25	28	52
2	8	8	8	Mod.	"	+	−	−	++	−	−	−	−		30	103	"	21	"	"	25	37	S.
3	8	8	8	"	"	−	−	−	++	−	−	−	−		20	73	"	21	"	"	25	26	57
4	8	8	8	Sev.	Sl.	−	−	−	++	−	−	−	−		18	29	50	14	"	"	17	24	31
5	8	8	8	Mod.	Sev.	−	−	−	−	−	−	−	−		30	S.	S.	21	80	"	19	27	S.
6	8	8	8	Sev.	"	−	−	−	−	−	−	−	−		17	"	41	10	54	"	12	27	51
7	8	8	8	Mod.	Sl.	−	−	−	+	−	−	−	−		17	29	50	12	80	"	10	51	S.
8	8	8	8	Sl.	Sev.	−	−	−	−	−	−	−	−		20	39	S.	22	44	"	24	29	43

* In the tables N. indicates none, Sl., slight, Mod., moderate, Sev., severe, and Inf., infiltration. The figures indicate the hours of survival of each mouse after injection. S. indicates survival over 140 hours.

to a reddening and swelling of the area from the shoulder almost to the wrist, persisting 3 days and rendering use of the arm during this time difficult. In three adults a chill with some nausea, marked malaise, and slight rise of temperature occurred in the first 24 hours. It was concluded that reactions of this type were too severe to be permissible in a large scale vaccination. A series of persons (Nos. 10 to 21, 25, and 27, Table III) was accordingly inoculated with four or five injections of much smaller doses at 3 to 7 day intervals (except

in two cases in which the interval between the first two doses was 2 days). Inasmuch as the suggestion had been made that Type III was especially prone to induce severe local reactions this type was omitted from one of the inoculations in Individuals 10 to 16 and 27. No difference could be observed between the local reaction in these cases when Type III was omitted. The dosage used in Individuals 12 to 16 was as follows:

Day.	No. of billions of Type I.	No. of billions of Type II.	No. of billions of Type III
1	1	1	1
4	2	2	2
7	4	4	4
13	6	6	

The dosage of each type in other individuals was as follows: Individual 17: first injection 1 billion; second injection 2 billion; third injection 3 billion; fourth injection 4 billion. Individuals 18 and 20: first injection 1 billion; second injection 2 billion; third injection 2 billion; fourth injection 3 billion. Individuals 19 and 21: first injection 1 billion; second, third, and fourth injections each 2 billion. Individual 25 received only half as much of Type III as of Types I and II at each injection. Of these fourteen adults, marked local reactions occurred after at least one of the inoculations in four instances but not severe enough to incapacitate the individual. In none of these instances was there any severe general reaction. These doses appeared to be such as could be satisfactorily employed on a large scale without unduly incommoding a command. Still smaller doses were employed in Individuals 32, 33, 36, 37, and 38. These were without any severe local reactions and with moderate or no general reaction.

The experimental work of Cole and Moore (5) showed that in rabbits an immunity could be more rapidly induced by small daily intravenous inoculations of antigen than by much larger intravenous inoculations at longer intervals. To test the applicability of this principle to subcutaneous injections in man, four adults (Nos. 39 to 42) were given daily subcutaneous injections of 1 billion cocci of each type for 7 days. These injections were associated with only the mildest local reactions and with no constitutional reaction. The

TABLE III.

Cases Receiving Multiple Doses of Varying Amounts.

Individual No.	No. of injections.	Intervals.	Total dosage in billions.			Reaction.		Agglutination.																Mouse protection.											
								Before vaccination.						8 days after last injection.										Before vaccination.			8 days after last injection.								
								Type I.		Type II.		Type III.		Type I.				Type II.				Type III.					Type I.			Type II.				Type III.	
								1:1	1:3	1:1	1:3	1:1	1:3	1:1	1:3	1:10	1:30	1:1	1:3	1:10	1:30	1:1	1:3	1:10			0.001 cc.	0.0001 cc.	0.001 cc.	0.0001 cc.	0.0001 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.001 cc.	0.0001 cc.
		days																																	
9	3	4	34	34	34	Mod.	Sl.	−	−	−	−	−	−	+		−	−	++		++	−	−		−	S.	S.	12	15	32	28	S.	S.	S.	S.	S.	32	S.
10	5	4–2	12	12	6	Sev.	"							±	±	−		±	±	±	−	−	−	−							"	"		"	"		24
11	5	4–2	12	12	6									±	±	+	−	+	±	±	−	−	−	−							24	"		"	"		24
12	4	6–3	13	13	7	Sl.	N.	−		−		−		++	++	+		±+	+	−		−			18	24	16	18	30	S.	S.	"	26	"	80		S.
13	4	5–3	13	13	7	Mod.	Sl.	−		−		−		±+	±+	±		+	+	−		−			19	24	22	24	37	43	"	"	13	117	S.		29
14	4	4–3	13	13	7	Sl.	N.	−		−		−		++	++	±		−	−	−		−			18	35	14	45	80	69	"	"	25	S.	"		28
15	4	5–3	13	13	7	Mod.	Sl.	−		−		−		−	+	−		−	−	−		−			19	19	17	15	34	37	65	"	S.	"	"		S.
16	4	6–3	13	13	7	Sl.	N.	−		−		−		−	−	−		−	−	−		−			39	26	29	21	23	18	37	"	21	68	"		27
17	4	5–3	10	10	10	"	"	−		−		−		−	+	−		±+	+	−		−			17	23	16	18	33	60	S.	"	16	S.	104		33
18	4	4–3	8	8	8	Mod.	Mod.							++	+	−		+	±	−		−	−	−												
19	4	4–3	7	7	7	Sev.	"							++	+	−		++	±+	−		−	−	−							S.	S.			S.		34
20	4	4–3	8	8	8	"	"							±	−	−		−	−	−		−	−	−													
21	4	4–3	7	7	7	Mod.	Sl.							−	−	−		−	−	−		−	−	−							14	17	20	17	41	"		27
																															14	26	53		65	"		66

| | | |

immune body response in the sera of these various groups is shown in Tables II and III.

From sixteen persons serum was obtained before commencing the vaccination. In none of these was there any agglutinin demonstrable against any of the three types and in only one instance (No. 9) were there any survivals in the mouse protection test, in this case against Type I. On the other hand, 8 days after the vaccination agglutinins to some extent were demonstrable in twenty-seven of the forty-two individuals studied and a definite degree of protective power as shown by the survival of at least some of the mice in all of the forty cases in which the mouse protection test was performed.

The degree of agglutinin production and of mouse protection varies so greatly in the individual cases in any one group that it becomes difficult to draw positive deductions as to the merits of the various methods of administration employed. This difficulty is indicated if we group the persons according to dosage and number of inoculations and tabulate the percentage of positive agglutinations at each titer and of mouse survivals for each dose of culture for these groups. This we have done in Table IV. From a study of this table the conclusion seems justified that the first group (Adults 10 to 21) which comprises those receiving the largest total dosage exhibits definitely the best response as judged by both agglutination and the mouse protection test. The second group (Adults 32, 33, 36, 37, and 38) received the smallest total dosage and gave definitely less satisfactory response. A comparison of the third group (Adults 1 to 8), the fourth group (Adults 18 to 21, 25, and 27), and the fifth group (Adults 39 to 42), all receiving practically the same total dosage but in the third group given in a single large dose, in the fourth in three or four moderate doses at 3 to 7 day intervals, and in the fifth in seven very small daily doses, shows no definite difference that can be detected in the response of the three groups. The conclusions that we draw from these experiments are that the immune response as measured by these tests will depend on the total dosage of vaccine and is little influenced by the number of doses into which this quantity is divided. The difference in toxic reaction, however, in these three groups was definite. The small daily doses gave hardly any reaction. The single very large dose gave rise to several severe local and constitutional reactions.

TABLE IV.

Summary by Percentages of Agglutination and Protection Tests in Sera.

Group.	Percentage of positive agglutinin reactions.						Percentage of mouse survivals in protection test.						
	Type I.			Type II.			Type I.			Type II.			Type III.
	1:1	1:3	1:10	1:1	1:3	1:10	0.01 cc.	0.001 cc.	0.0001 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.0001 cc.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Group 1* (Individuals 10 to 21).....	67	75	33	58	58	17	12	54	82	14	60	82	18
" 2 (" 32, 33, and 36 to 38).....	40	20	0	20	20	0	0	25	75	0	25	75	25
Group 3 (Individuals 1 to 8).....	25		0	62		0	12	37	62	0	50	100	37
" 4 (" 18 to 21, 25, and 27).....	50	50	0	33	33	0	0	40	40	0	25	100	0
Group 5 (Individuals 39 to 42).....	0	25	25	25	25	0	0	50	50	0	67	75	0
" 6 (" 22 " 24, 26, 28 to 31, 34, and 35).....	67	44	0	44	22	11	11	40	90	10	50	70	20

* Group 1 received a total dosage of from 7 to 13 billion cocci of each type. Group 2 received a total dosage of from 2½ to 4 billion cocci of each type. Groups 3, 4, and 5 received a total dosage of from 7 to 9 billion cocci of each type, Group 3 in a single large injection, Group 4 in three or four moderate injections at 3 to 7 day intervals, and Group 5 in seven small daily injections. Group 6 received a total dosage of 3 to 6 billion cocci of each type and developed infiltrations.

Vaccinations of Troops.

On the basis of these preliminary experiments we adopted for the larger scale vaccinations a moderate total dosage of 6 to 9 billion cocci of each type and administered this in four small doses at weekly intervals. By this method we expected to secure a definite immune response in the vaccinated individuals with a minimum of severe reactions and without undertaking a larger number of injections than would be within the capacity of the medical staff of the camp to carry out.

The detailed dosage decided upon was:

1st inoculation:	Pneumococcus Type I	1	billion
	" " II	1	"
	" " III	1	"
	Total.....	3	"
2nd inoculation:	Pneumococcus Type I	2	billion
	" " II	2	"
	" " III	2	"
	Total.....	6	"
3rd inoculation:	Pneumococcus Type I	3	billion
	" " II	3	"
	" " III	1½	"
	Total.....	7½	"

4th inoculation: Same as 3rd inoculation.

The vaccine was made up in three different concentrations so that the dose in every case was 0.5 cc.

Altogether, 12,519 men were vaccinated, about 40 per cent of the mean strength of the command. A great majority of these received three or four inoculations. Some, however, had only one or two. The vaccine was given at 5 to 7 day intervals, except in one organization where unavoidable circumstances necessitated a lapse of 20 days between the first and second inoculations. In most cases the vaccine was administered in the arm.

Reactions.

The constitutional reactions to the pneumococcus inoculations were usually negligible. Out of the entire number vaccinated, only twenty-five men were sufficiently ill to remain in quarters or the hospital for a short time. This number would probably have been larger if the vaccination had been compulsory. As it was, those who were upset by the first or second inoculation were usually not given the third or fourth. The impression prevailed among the regimental surgeons that the reactions to pneumococcus vaccination were milder than those to typhoid vaccination. The troops, however, were in better physical condition when they received the pneumococcus vac-

cine than when the typhoid inoculations were given. In those who reacted severely the symptoms simulated an attack of influenza. The patient complained of general malaise, chilly sensations, fever, and muscular pains. In addition, a certain number of those who reacted severely had symptoms referrable to the upper respiratory tract, such as coryza, sore throat, cough, and pain in the chest. A number of those who were suffering from infections of the air passages at the time of inoculation, claimed that their symptoms were

TABLE V.

Incidence of Infiltrations and General Reactions among the Vaccinated Troops.

Organization.	No. vaccinated (approximate).	Reactions.	
		Infiltrations.	General reaction (quarters or hospital).
305th Infantry	2,500	43	12
306th "	2,800	62	6
307th "	1,500	6	0
308th "	2,150	28	5
302nd Sanitary Train	700	3	1
367th Infantry (colored)	1,500	1	0
304th Machine Gun Battalion	400	2	0
305th " " "	400	4	1
306th " " "	500	3	0
302nd Engineers	35	0	0
Base Hospital	25	0	0
Total	12,510	152 (1 in 82)	25 (1 in 500)

more marked after receiving the injection. Constitutional reactions to pneumococcus vaccination apparently develop more slowly than with typhoid inoculation, sometimes not appearing until 24 hours after the injection.

The local reaction to pneumococcus vaccination differs little, as a rule, from that to typhoid vaccination. At the point of inoculation an area of tenderness and induration develops, usually about 5 to 10 cm. in diameter. The area of induration is nearly always oblong and extends down the arm below the point of inoculation. The axillary glands are sometimes swollen and tender. The tenderness

and swelling at the point of inoculation rapidly decrease, however, and at the end of 3 or 4 days have usually disappeared.

An unexpected and somewhat troublesome complication arose in connection with the vaccination which at first gave us some concern; this was the development of a certain number of small infiltrations at the site of inoculation. They developed slowly, rarely coming to the stage of fluctuation before the 6th or 7th day after inoculation. In size they were usually 2 or 3 cm. in diameter, and in only one instance extended down deeper than the subcutaneous tissue. At first they were looked upon as the result of careless technique, but repeated cultures showed them to be invariably sterile. Some of them developed after the first inoculation, but a greater number followed the larger doses. In a few cases an infiltration developed with each inoculation given. This would have probably happened more frequently had the vaccination not been discontinued in those who developed the condition after the first or second dose. At first the regimental surgeons made an incision, but later it was found that the infiltrations would progress favorably if left alone. Altogether, 152 men developed the lesion (1 in every 82, Table V). They were fairly evenly distributed throughout the various organizations vaccinated, with the exception of the 1,500 vaccinated negroes, among whom only one developed. The infiltrations were tender and painful in the early stage of their development but later became cold and painless. They apparently developed from a hypersensitiveness to the pneumococcus or pneumotoxin. In the hope of discovering whether these individuals exhibited a more or less marked immune response in their sera, the sera of ten such persons were studied (Table III, Adults 22 to 24, 26, 28 to 31, 34, and 35). The percentile results are tabulated in Table IV as Group 6. Apparently the immune response in this group is analogous to that of the other individuals receiving the same total dose of vaccine. We would conclude, therefore, that there is no demonstrable difference in the degree of immune response by the tests we have used in individuals showing pronounced local reaction even to the extent of local infiltration described. Few of the men responding in this way had ever had pneumonia. With only one exception they did not occur among the colored troops who are especially susceptible to pneumonia. They

are not related in any way to the method of administration of the vaccine.

In order to test the relation of these severe reactions to the susceptibility to pneumotoxin, the following experiments were performed. Pneumotoxin was prepared by Cole's (6) method, which consists of growing pneumococci in plain broth; centrifuging and washing in normal saline solution; solution of the pneumococci in weak sodium taurocholate solution at 37°C.; dilution to one-tenth the bulk of the original broth culture. This pneumotoxin was diluted 1: 10 with normal saline solution and injected intradermally to the amount of 0.1 cc. in a series of healthy volunteers. Little resulting reaction was noted in most individuals. However, in those who had shown severe local reactions to the vaccination or had developed infiltrations, an extensive areola developed about the pneumotoxin injection, reaching the maximum after about 24 to 36 hours and often being associated with considerable tenderness. The reaction to the pneumotoxin occurred regardless of the type used in its preparation, exhibiting no specificity for type. These findings suggest that the severe local reactions to the vaccine are due to an unusual sensitiveness to a pneumotoxin which is common to all the types of pneumococci.

Table V shows the number of men vaccinated in each organization and the incidence of infiltrations and severe general reactions in each.

Bacteriological Examination of Sputa.

Particular attention was directed to the bacteriological examination of the sputum in the cases of pneumonia that developed during the period of observation. In seven instances this examination, through some unavoidable circumstance, was not completed. In all the others, however, the sputum was examined and the predominant organism determined.

The Avery blood broth method (7) was used in a great majority of cases and in addition, whenever it was possible, a mouse was inoculated, or a direct culture from sputum was made on a blood agar plate. The following figures indicate the number of times each method was used:

1. Both mouse and blood broth..... 47
2. " blood broth and direct culture..... 23
3. Blood broth alone..... 108
4. Mouse alone..... 5

Results of Vaccination.

The vaccination of the troops began on February 4, 1918. The Division was transferred from Camp Upton about April 15, 1918. The following figures are based on the period extending from February 4 to April 15, about 10 weeks. The number of troops vaccinated was 12,519. The number of unvaccinated was approximately 19,481. The latter figure varied, of course, from day to day as new men came and others departed. The vaccinated men were in stable organizations where the personnel underwent little change.

Before discussing the results of vaccination, it is of interest to note the incidence of pneumonia previous to the beginning of vaccination in the various organizations which later received the vaccine and to compare this with the incidence of pneumonia at the same period among the organizations which did not later receive the vaccine. Previous to February 4, the day on which the experiment began, there had been 91 cases of pneumonia among the troops at Camp Upton. Of these, 29 occurred among the organizations which were subsequently vaccinated, while 43 cases occurred among the units which were later to be used as a control; 19 occurred among casuals not included later in either group. It will be seen from this that the cases of pneumonia were quite evenly divided between the 40 per cent of the troops which were to be vaccinated and the 60 per cent which were to be used for controls. Of the 29 pneumonias occurring among the organizations which were subsequently vaccinated, 9 were due to pneumococci of Type I, II, or III and 11 to Pneumococcus Type IV. Of the 43 pneumonias occurring among the control group, 16 were due to pneumococci of Type I, II, or III and 17 to Pneumococcus Type IV.

Furthermore, about 30 per cent of all these pneumonias were streptococcus cases and these, too, were fairly equally divided between the two groups of organizations, 9 occurring among the men vaccinated later, and 10 among the control group.

Incidence of Pneumonia among the Vaccinated Troops from February 4 to April 15, 1918.—There has been but one case of pneumonia due to pneumococcus of Type I, II, or III among the vaccinated troops during this period. This case, due to Type I pneumococcus, developed 24 hours after the first inoculation and therefore before protection could have been developed. During this period sixteen cases of other types of pneumonia occurred among the vaccinated troops (Table VI). There were nine Type IV pneumococcus cases; three of these had only received one injection of vaccine. The remaining seven cases were streptococcus infections, six due to the hemolytic streptococcus, and one to *Streptococcus viridans*. None of the pneumococcus cases died. The Type I case received Type I serum and

TABLE VI.

Incidence of Pneumonia among the Vaccinated Troops, February 4 to April 15, 1918.

Average strength of command, Feb. 4 to Apr. 15, 1918	32,000
No. of troops vaccinated against pneumonia	12,519 (40 per cent)
" " unvaccinated men (average)	19,481 (60 " ")
Incidence of pneumonia among the vaccinated troops, Feb. 4 to Apr. 15:	
Pneumococcus Type I (developed 24 hrs. after 1st injection)	1
" " IV (3 cases receiving only 1 injection)	9
Streptococcus hæmolyticus	6
" *viridans*	1
Total No. of pneumonias among the vaccinated troops	17

made an uneventful recovery. Most of the Type IV cases ran a mild course, so mild in some cases that the diagnosis of pneumonia was made only by the aid of the x-ray. Two of the streptococcus cases died, both being of the hemolytic group.

Incidence of Pneumonia among the Unvaccinated Troops from February 4 to April 15, 1918.—The unvaccinated fraction of the camp has been divided into two groups (Table VII). First, the old troops whose physical condition and resistance to infection were presumably the same as that of the vaccinated men. They constituted, numerically, about 75 per cent of the controls. Second, the new troops who consisted of newly drafted men coming into camp between February 26 and April 15 and who by reason of their lack of training were prob-

ably more susceptible to infection than the seasoned troops. These comprised about 25 per cent of the controls. Among the old troops there were eighteen cases of pneumonia due to Pneumococcus Type I, II, or III, and seventeen cases due to Type IV, making a total of

TABLE VII.

Incidence of Pneumonia among the Unvaccinated Troops, February 4 to April 15, 1918.

Type of pneumonia.	No. of cases of pneumonia		
Old troops (75 per cent of control).			
Pneumococcus Type I	8		
" " II	5	18	
" " III	5		35
" " IV	17	17	
Streptococcus hæmolyticus	45		
" *viridans*	23		68
B. influenzæ	1		
Type undetermined	6		
Total	110		
New drafted men, Feb. 26 to Apr. 15 (25 per cent of control).			
Pneumococcus Type I	2		
" " II	4	8	
" " III	2		24
" " IV	16	16	
Streptococcus hæmolyticus	27		
" *viridans*	11		38
Type undetermined	1		
Total	63		

Total No. of Type I, II, and III pneumonias among the unvaccinated troops	26
" " " " I, II, III, and IV " " " " " "	59
" " " streptococcus pneumonias among the unvaccinated troops	106
" " " pneumonias among the unvaccinated troops	173

thirty-five cases of pneumococcus pneumonia. There were sixty-eight streptococcus cases, forty-five of the hemolytic type and twenty-three of the non-hemolyzing type. There was one *Bacillus influenzæ* case and six cases in which the type was not determined.

Among the new men the proportion was about the same. There were eight cases due to Pneumococcus Type I, II, or III. There were sixteen cases of pneumonia due to Pneumococcus Type IV, making twenty-four cases of pneumococcus pneumonia. There were thirty-eight cases of streptococcus pneumonia, twenty-seven of the hemolytic type and eleven of the non-hemolyzing type. There was one case in which the organism was not determined.

TABLE VIII.

Mortality Rates.

Type of pneumonia.	Deaths.	
Among unvaccinated troops.		
		per cent
Pneumococcus.		
Types I, II, and III	7	27
Type IV	6	18
Streptococcus hæmolyticus	26	36
" *viridans*	4	12
Type undetermined	5	
Total	48	28
Annual pneumonia death rate per 1,000 for unvaccinated troops	12.8	
Among vaccinated troops.		
Pneumococcus	0	
Streptococcus hæmolyticus	2	
" *viridans*	0	
Total	2	11.7
Annual pneumonia death rate per 1,000 for vaccinated troops	0.83	

A summary of the control shows that there were twenty-six cases of pneumococcus pneumonia of Type I, II, or III. There were thirty-three Pneumococcus Type IV cases, making a total of fifty-nine pneumococcus pneumonias. There was a total of 106 streptococcus pneumonias, 72 of which were of the hemolytic type and 34 of the *viridans*, or non-hemolyzing type. Altogether there were 173 pneumonias among the unvaccinated troops.

There were seven deaths among the Pneumococcus Type I, II, and III cases, or 27 per cent (Table VIII). There were six deaths among the Pneumococcus Type IV cases, or 18 per cent. Of the streptococcus cases, hemolytic type, twenty-six died, or 36 per cent. There were only four deaths among the *viridans* cases, the mortality being 12 per cent.

DISCUSSION.

The first and most important deduction to be made from these statistics is that pneumococcus pneumonia of Types I, II, and III has not occurred among the vaccinated troops, whereas twenty-six cases have occurred among the unvaccinated part of the camp. This is the best test of the value of the vaccination as a prophylactic measure. The one case of Type I pneumonia that developed 24 hours after the patient had received his first inoculation may properly be excluded from among the vaccinated cases, as the patient was probably already infected at the time he received the injection and could not have had time for the development of any appreciable immunity.

As Lister pointed out, the diminution or disappearance of certain types of pneumonia as the result of specific type inoculation is a more critical test of the efficacy of pneumonia prophylaxis than the mere simultaneous comparison of pneumonia rates in vaccinated and unvaccinated groups. It is true that the period of observation in this experiment has been short, but the immunity produced by the vaccine appears to have been adequate for this period of time. How much longer this immunity will last can only be determined by following these men and studying the cases of pneumonia that subsequently develop among them.

There have been only six cases of Pneumococcus Type IV pneumonia among the men who received two or more injections of the vaccine, while the control column shows thirty-three cases among the unvaccinated troops. The marked difference in these figures and the mild course which the Pneumococcus Type IV pneumonias ran in the vaccinated series might suggest that some cross-protection against Type IV pneumococcus has been afforded by the Type I, II, and III vaccine. This theory, however, is hardly admissible when we note that the same difference occurs in the incidence of streptococcus pneumonias

in the vaccinated and the unvaccinated troops. There were 106 cases of streptococcus pneumonia among the unvaccinated troops, whereas there were only seven streptococcus pneumonias among the vaccinated troops. Among the 3,500 colored troops, half the companies were vaccinated against pneumonia, the other half were not. There were twenty-eight cases of streptococcus pneumonia among the unvaccinated half and only two cases of streptococcus pneumonia among the vaccinated half, yet these men were living in the same part of the camp and closely associated on drill-grounds, and in recreation and amusement halls.

We have no explanation to offer for this difference. That an epidemic of streptococcus pneumonia occurred at about this time in the camp, there can be no question. Not only the bacteriological studies, but the clinical course, the frequency of empyema, the mortality rate, and the character of the autopsy findings confirm the bacteriological diagnosis. Why this epidemic should to a large extent have spared the vaccinated troops cannot be explained. The incidence of pneumonias, pneumococcic and streptococcic, was approximately equally distributed in the two groups of organizations previous to the beginning of the vaccination. While no explanation of this phenomenon can be offered, it presents no argument against the use of prophylactic vaccination against the pneumococcus, nor does it in the slightest degree weaken the importance of the fact that while twenty-six cases of Type I, II, or III pneumococcus pneumonia occurred among the unvaccinated, none occurred among the vaccinated troops. While it is still too early to draw final conclusions from this experiment, we feel that the results are sufficiently encouraging to justify further investigation along these lines.

SUMMARY.

1. From a study of the agglutinins and protective power of the serum of 42 persons vaccinated against the pneumococcus, Types I, II, and III, it is demonstrated that a definite immune response has been secured to Types I and II by the dose of vaccine employed. Little evidence of response to Type III can be demonstrated by these methods, but this is of less significance in that in animals it is rela-

tively difficult to secure antibodies against this strain in the serum, even though a considerable degree of active immunity may have been produced in the vaccinated animal.

2. The degree of response to the vaccination appears to be dependent upon the total dosage of each type of pneumococcus administered. While some response may be elicited by $2\frac{1}{2}$ billion cocci of each type, a much more constant and greater response follows 13 billion.

3. In subcutaneous administration the manner in which the total dosage is divided, whether given in a single large dose, in seven small daily doses, or in three to five moderate doses at 3 to 7 day intervals, seems to have little influence upon the degree of immune response, provided the total dosage is the same.

4. The local and general toxic reaction varies greatly in different individuals. The smaller the individual doses, the fewer are the severe reactions. This makes it desirable to divide the total dosage into as many inoculations as circumstances make practicable.

5. At Camp Upton 12,519 men have been vaccinated against Pneumococcus Types I, II, and III. Three or four doses were given at intervals of 5 to 7 days with a total dosage of 6 to 9 billion of Types I and II and $4\frac{1}{2}$ to 6 billion of Type III.

6. During the 10 weeks that have elapsed since the vaccination, no cases of pneumonia of these three types have occurred among the men who had received two or more injections of vaccine.

7. In a control of approximately 20,000 men there were twenty-six cases of Pneumococcus Types I, II, and III pneumonias during the same period.

8. The incidence of Pneumococcus Type IV pneumonia and streptococcus pneumonia was much less among the vaccinated troops than among the unvaccinated. No explanation has been advanced for this difference.

9. Small sterile infiltrations disappearing spontaneously occasionally follow the injection of large doses of pneumococcus vaccine and appear to be an expression of cutaneous hypersusceptibility.

10. The persons who develop these lesions exhibit local reactions to each dose of vaccine. They also give abnormally marked reactions to intradermal injections of pneumotoxin. They do not, however,

exhibit anything notable in the agglutinative or protective powers of their sera after vaccination. Whereas the immune response is characteristically specific for the type of pneumococcus, this reaction is not specific for any type. We have found no evidence that Type III is more prone to elicit these severe local reactions than are Types I and II.

11. Prophylactic vaccination against pneumococcus of Types I, II, and III is practical and apparently gives protection against pneumonia produced by these types. It remains to be determined how long this immunity persists.

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A HOMOHEMOLYTIC SYSTEM FOR THE SERUM DIAGNOSIS OF SYPHILIS.

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There is little doubt that the elimination of the use of guinea pig complement from the serodiagnostic procedure is a great advance towards the simplification of this important reaction, for that element has to be used while it is perfectly fresh, necessitating the maintenance of guinea pigs in the laboratory. Attempts to preserve the activity of the complement, either by means of desiccation[1] or hypertonization,[2] have been only partially successful, and it deteriorates in a comparatively short time. The best preservative for guinea pig complement is that recommended by Rhamy,[3] who found that sodium acetate in appropriate concentration keeps the complement active for several weeks.

Some years ago Hecht[4] proposed the utilization of the natural anti-sheep hemolysin and complement in the fresh serum of patients, but the difficulty in this method lies in the fact that in some sera there is no natural hemolysin or too little to produce hemolysis, even in the control tubes without the antigen. On the other hand, some sera contain a considerable excess of the natural anti-sheep hemolysin. Granting that the insufficiency of the natural anti-sheep hemolysin can be remedied by adding an adequate quantity of a non-syphilitic

[1] Noguchi, H., On the influence of the reaction and of desiccation upon oposonins, *J. Exp. Med.*, 1907, ix, 455; Serum diagnosis of syphilis, and the butyric acid test for syphilis, Philadelphia and London, 1st edition, 1910.

[2] Austin, F. D., A new method for preserving complement for making the Wassermann or Noguchi blood-test, *J. Am. Med. Assn.*, 1914, lxii, 868.

[3] Rhamy, B. W., Preservation of complement. A preliminary report, *J. Am. Med. Assn.*, 1917, lxix, 973.

[4] Hecht, H., Eine Vereinfachung der Komplementbindungsreaktion bei Syphilis, *Wien. klin. Woch.*, 1909, xxii, 338.

serum containing enough hemolysin, the test still calls for the washed sheep corpuscles. In other words, having eliminated the use of guinea pig serum as complement, we still have to depend upon the sheep corpuscles for an indicator of hemolysis.

In ordinary times there should be no difficulty in obtaining the guinea pig complement or sheep blood corpuscles, and it would be immaterial whether one used the anti-sheep hemolytic system or the anti-human hemolytic method as advocated by the writer[5] and accepted by many serologists, including those in the United States Army and Navy. Perhaps it may not be amiss to call attention here to the fact that the anti-human hemolytic system of the writer is not limited in its use to unheated sera but applies equally to inactivated sera, the only differences between the examination of fresh and inactivated sera being that only 0.02 cc. of the former is required, instead of 0.08 to 0.1 cc. of inactivated specimens, and that in the case of inactivated sera not only the acetone-insoluble fraction of tissue lipoids, but also any properly titrated alcoholic extracts may be used as the antigen.[6] With fresh sera emphasis has been placed on the fact that the crude alcoholic extracts or those alcoholic extracts to which cholesterol has been added are to be avoided, as in this combination there is apt to occur a non-specific proteotropic complement fixation.[7] It is therefore essential that only the acetone-insoluble fraction of tissue lipoids should be used in combination with fresh sera. With these points in view there should be no confusion as to the extent of applicability of the test to fresh and inactivated sera.[6]

There is another possibility, namely that by eliminating the use of the guinea pig complement from the anti-human hemolytic system the technique and the material for the serodiagnosis of syphilis can be greatly simplified. That this is the case is shown below. Efforts to introduce similar methods have already been made by Tscher-

[5] Noguchi, A new and simple method for the serum diagnosis of syphilis, *J. Exp. Med.*, 1909, xi, 392.

[6] Noguchi, Serum diagnosis of syphilis, and luetin reaction, together with the butyric acid test for syphilis, Philadelphia and London, 3rd edition, 1912.

[7] Noguchi, On non-specific complement fixation, *Proc. Soc. Exp. Biol. and Med.*, 1909–10, vii, 55.

nogubow,[8] Emery,[9] Butler and Landon,[10] Myer,[11] and Thompson,[12] all employing human complement. A brief review of these methods will be given later.

Principle of the Method.

It is well known that complement is present in every fresh serum and that the quantity may sometimes vary. In the serodiagnosis of syphilis, whether by the anti-sheep system of Wassermann or the anti-human system of the writer, the serum of the guinea pig is chosen because of its richness in complement and also because guinea pig complement is probably more readily fixed[13] by the antigen-antibody combination than the sera of other animals, such as the horse, rabbit, sheep, pig, etc.[14, 15] As to the complement in fresh human sera, there are not many data regarding its action upon human corpuscles. It is understood that human sera exert no hemolytic action upon human corpuscles, especially when there is no isohemolysin. But upon the addition of a sufficient quantity of the anti-human hemolytic amboceptor the complement dissolves the human corpuscles just as guinea pig complement does when added to the suspension of human corpuscles and the specific anti-human hemolytic amboceptor.

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[13] Noguchi, H., and Bronfenbrenner, J., Variations in the complementary activity and fixability of guinea pig serum, *J. Exp. Med.*, 1911, xiii, 69.

[14] Noguchi, Non-fixation of complement, *Proc. Soc. Exp. Biol. and Med.*, 1909–10, vii, 14.

[15] Noguchi and Bronfenbrenner, The comparative merits of various complements and amboceptors in the serum diagnosis of syphilis, *J. Exp. Med.*, 1911, xiii. 78

The only difference between the action of the human and the guinea pig complement lies in the fact that the former requires more anti-human hemolytic amboceptor to render it active against the human corpuscles than the latter. The relative lytic values of the human and the guinea pig complement are shown in the following experiments.

Comparative Complement Values of the Human and the Guinea Pig Sera Relative to the Anti-Human Hemolytic Amboceptor and Human Corpuscles.

0.04 cc. of guinea pig complement was put in each of a number of tubes, together with 1 cc. of a 1 per cent suspension of human corpuscles. Varying quantities of the anti-human hemolytic amboceptor (immunized rabbit) were added to the tubes and the results read after 30 minutes at 37°C. in a water bath thermostat. The results are shown in Table I. The experiment shows the titer of the anti-

TABLE I.

Titration of the Anti-Human Amboceptor with Guinea Pig Complement.

Amount of serum to each tube.*	Anti-human hemolytic immune serum.	
	No. 633 (rabbit).	No. 634 (rabbit).
cc.		
0.01	Complete hemolysis.	Complete hemolysis.
0.007	" "	" "
0.005	" "	" "
0.004	" "	" "
0.003	" "	" "
0.002	" "	" "
0.0015	" "	" "
0.001	" "	" "
0.0007	" "	" "
0.0005	Partial hemolysis.	" "
0.0004	No "	Partial hemolysis.
0.0003	" "	No "
0.0002	" "	" "
0	" "	" "

* Each tube contained guinea pig complement 0.04 cc. and 1 per cent human corpuscle suspension 1 cc.

human hemolytic immune serum No. 633 to have been 0.0007 cc. and that of No. 634, 0.0005 cc. in the presence of 0.04 cc. of guinea pig complement. 0.1 cc. of the guinea pig serum alone was itself somewhat hemolytic.

From the results recorded in the first part of Table II it appears that in the presence of 0.1 cc. of a fresh human serum at least 0.005

TABLE II.

Titration of the Anti-Human Amboceptor with Human Complement.

Serum 3 + amboceptor.*	Result.
cc. *cc.*	
0.1 + 0.1	Complete hemolysis.
0.1 + 0.05	" "
0.1 + 0.03	" "
0.1 + 0.02	" "
0.1 + 0.01	" "
0.1 + 0.005	" "
0.1 + 0.003	Considerable "
0.1 + 0.002	No "
0.1 + 0.001	" "
0.5 + 0.02	Complete hemolysis.
0.3 + 0.02	" "
0.2 + 0.02	" "
0.1 + 0.02	" "
0.05 + 0.02	Slight hemolysis.
0.02 + 0.02	No "

* Human serum as complement. 1 per cent human corpuscle suspension 1 cc. in each tube.

cc. of the anti-human amboceptor No. 633 was needed to produce complete hemolysis; that is, about seven times the amount required in the case of guinea pig complement. In other words, the activity of human complement is nearly one-seventh that of the guinea pig serum.

In the second part of the table it is shown that 0.05 to 0.02 cc. of the same fresh human serum was unable to cause complete hemolysis even in the presence of 0.02 cc. of the amboceptor No. 633, which is an equivalent of thirty minimal lytic doses when calculated on the basis of 0.04 cc. of the guinea pig complement. It is evident that

the amount of amboceptor required to dissolve the human corpuscles in the presence of human complement is many times that necessary with guinea pig complement.

The next point is to determine whether or not average fresh human serum contains enough complement to produce complete hemolysis. Upon this fact depends the possibility of utilizing the human com-

TABLE III.

Quantitative Relation between Human Complement and Anti-Human Amboceptor.

Serum No. (48 hrs. old).*	Human serum 0.1 cc.		Human serum 0.05 cc.	
	Amboceptor 0.005 cc.	Amboceptor 0.01 cc.	Amboceptor 0.005 cc.	Amboceptor 0.01 cc.
1	Considerable hemolysis.	Complete hemolysis.	No hemolysis.	Complete hemolysis.
2	No hemolysis.	No hemolysis.	" "	No hemolysis.
3	Considerable hemolysis.	Complete hemolysis.	" "	Complete hemolysis.
4	" "	" "	" "	" "
5	" "	" "	" "	" "
6	Complete hemolysis.	" "	" "	" "
7	Considerable hemolysis.	" "	" "	" "
8	No hemolysis.	No hemolysis.	" "	No hemolysis.
9	" "	" "	" "	" "
10	Considerable hemolysis.	Complete hemolysis.	" "	Complete hemolysis.
11	Almost complete "	" "	" "	" "
12	Considerable "	" "	" "	" "
13	Complete hemolysis.	" "	" "	" "
14	Considerable hemolysis.	" "	" "	" "
15	" "	" "	" "	" "
16	" "	" "	" "	" "
17	" "	" "	" "	" "
18	" "	" "	" "	" "
19	" "	" "	" "	" "
20	" "	" "	" "	" "

* Each tube contained 1 per cent washed human corpuscle suspension 1 cc.

plement in the serodiagnosis of syphilis. Twenty sera were tested for this purpose. All except three of them (Nos. 2, 8, and 9, Table III) contained sufficient complement in 0.05 to 0.1 cc. to cause complete hemolysis in the presence of 0.01 cc. of the anti-human amboceptor No. 633. No hemolysis occurred, however, in any tube containing 0.005 cc. of the amboceptor and 0.05 cc. of human serum.

There was considerable, and in some cases complete hemolysis in the tubes containing the same amount of the amboceptor but 0.1 cc. of the human serum.

For comparison these twenty sera were also tested upon sheep corpuscles (1 cc. of a 1 per cent suspension) for their natural anti-sheep hemolysin. The results obtained show that 0.05 cc. and 0.1 cc. both dissolved the sheep corpuscles in all except the three specimens (Nos. 2, 8, and 9) which failed to dissolve the human corpuscles in the presence of anti-human amboceptor. This means that there were at least three sera out of twenty which did not contain sufficient complement.

Out of 1,250 specimens of fresh human sera so far examined, 1,157 contained enough complement to produce complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in a dose of 0.1 cc., with the addition of 0.01 cc. of anti-human amboceptor (one unit), within a period of 20 to 30 minutes at 37°C. in a water bath thermostat. 72 specimens caused a partial hemolysis, and 21 no hemolysis.

Additional amboceptor in doses of from one-half to one ordinary minimal hemolytic unit to the partially hemolyzed tubes produced complete hemolysis on a further incubation of 15 minutes or longer. In the case of the sera, however, which showed no complementary action in the first combination, the addition of an extra quantity of the amboceptor caused only a tardy partial hemolysis or no hemolysis. From the serological standpoint we encounter at least three groups of human sera, those which contain the average amount of complement, those which contain a subnormal quantity (hypocomplementosis), and those which contain no complement (acomplementosis). Occasionally one meets with a fourth group in which the activity of the complement is unusually strong (hypercomplementosis). It is highly important to take these facts into account in testing human sera by the Bordet-Gengou reaction or the Wassermann reaction in syphilis.

The next step was devised in order to determine whether the insufficiency of complement could not be supplemented by the addition of an adequate quantity of fresh active serum. That this is easily accomplished was soon proved.

Mode of Utilization of Human Complement for the Serodiagnosis of Syphilis.

The experiments discussed above made it evident that in the majority of fresh human sera there is sufficient complement to cause a complete and prompt hemolysis of human corpuscles in the presence of an adequate quantity of the specific anti-human hemolytic amboceptor. Moreover, in cases where there is not enough complement, an active human serum may be added as a supplement. In fact, we are now in a position to produce complete hemolysis, and from this point it is only another step to test the presence or absence of a complement-fixing principle in a given specimen of human serum. One merely measures out a definite amount of the suspected serum into two tubes and then adds to one of the two an adequate amount of the antigen suspension. Both tubes are incubated for 30 minutes at 37°C. in a water bath; then the human corpuscular suspension and the anti-human hemolytic amboceptor are introduced into both tubes and the contents are well mixed by shaking. The tubes are once more incubated for 30 minutes at 37°C., and then after another 30 minutes or so at room temperature the result is read. It is necessary to shake the tubes two or three times during the incubation. No result should be taken as final unless the control tube without the antigen shows complete hemolysis. If hemolysis is incomplete at the end of the period indicated, adequate modification, which will be described later, must be made.

Procedure for the Examination of Human Sera Not More than 48 Hours Old.

It must be made clear in the beginning that specimens should be examined as soon as practicable, preferably within 24 hours after they are withdrawn from the patients. After 48 hours, even when the specimens are kept in the refrigerator, the complement gradually disappears from the serum. Sera kept for more than 3 days in a refrigerator must be tested by a special technique, to be given later. Specimens tinged deeply with hemoglobin give unsatisfactory results and should be rejected. Table IV indicates the amounts of reagents to be used in the test for fresh human sera and other details regarding it. The entire set of tubes should be duplicated for each specimen.

TABLE IV.

Procedure for Examining Fresh Human Sera.

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Patient's serum (fresh) 0.2cc. Antigen 0.1 " 0.9 per cent saline solution 1 "	First incubation at 37°C. for 30 min. in water bath or 1 hr. in air thermostat.	Both tubes receive 0.1 cc. of anti-human amboceptor, representing 1 hemolytic unit, and 0.1 cc. of 10 per cent human corpuscular suspension. Total volume 1.5 cc. Contents are well mixed by shaking.	Second incubation at 37°C., same as first, except that tubes are shaken three times during the period.	Reading of results after tubes have stood 30 min. at room temperature.
Control tube (back row).	Patient's serum (fresh) 0.2 " Antigen omitted. 0.9 per cent saline solution 1.1 "				

Positive and Negative Controls.

As in any other serodiagnostic procedure, each serum tested must be accompanied by a positive and a negative serum in order to control the reliability of the reagents. In a well appointed laboratory, where many tests are being made daily or every other day, the necessary positive and negative sera will be furnished by the tests of the previous occasion.

Varieties of Irregular Reactions and Their Adjustment.

Reference has already been made to the possible deficiency of complement in certain specimens of fresh human sera. With these hypocomplementary sera hemolysis in the control tubes without antigen proceeds slowly and remains incomplete at the end of the second incubation. To these sets of tubes, both the determinative and the control, an additional hemolytic amboceptor unit is introduced in order to reinforce the hemolytic activity of the complement. In cases in which hemolysis is marked only one-half the unit is added, but when hemolysis is slight a whole unit of the amboceptor is needed. The tubes should then be put in another rack and subjected to further incubation until complete hemolysis occurs in the control tubes (without the antigen). 15 minutes or longer may be required. Specimens which fail to hemolyze or in which hemolysis is incomplete, even with the additional amboceptor should be tested again by adding to them a quantity of fresh serum which has been shown to contain an average complement and at the same time to be devoid of any syphilitic fixation substance (negative serum). In these cases 0.1 cc. of the complementary serum is used with 0.2 cc. of the acomplementary serum. The mixture is then tested in the same way that any fresh serum is tested.

Procedure for the Examination of Human Serum More than 48 Hours Old.

As previously stated, specimens of human serum which have stood in a refrigerator longer than 48 hours are inconstant in their complementary activity, and many are markedly deficient. As a rule

sera not more than 72 hours old which have been kept constantly in the refrigerator at 4–6°C. still contain enough complement to make the test possible. It is best, however, to inactivate all sera whose complementary activity is no longer a certain factor, supplementing them with active human complement from negative fresh sera. If inactivation is complete (55°C. for 30 minutes), the sera are rendered free of the remnant of their native complement, and what is added later is of known and uniform quantity. 0.1 cc. of the complement serum (previously tested) is added to 0.2 cc. of the inactivated serum and the mixture then tested like any fresh serum. Table V gives the details of the procedure.

Procedure for the Examination of Cerebrospinal Fluids.

This procedure is comparatively easy and gives an entirely satisfactory result. It differs from that used for inactivated sera only in one respect, that of the quantity of the specimen used, which may vary from 0.2 to 0.5 cc. No inactivation is required, as the cerebrospinal fluid contains no complement, and 0.1 cc. of active negative human serum (previously tested) is added as complement (Table VI).

A Method of Preserving the Complement of Fresh Human Serum by Means of Sodium Acetate.

Rhamy[3] first observed that guinea pig complement remains active for a long time when mixed with sodium acetate in a strength of approximately 6 per cent of the acetate in the mixture. He recommends mixing 4 parts of the complement with 6 parts of a 0.9 per cent sodium chloride solution containing 10 per cent sodium acetate.

Human complement can also be kept active for some time by adding sodium acetate in a similar proportion. Specimens of human serum which have been mixed with the acetate can be satisfactorily tested after several days without the aid of complement from another source, since the complement remains active for at least 4 days at room temperature (18°C.). Therefore any serum which cannot be examined within 48 hours may be mixed with the acetate saline solution while perfectly fresh. If a good refrigerator is not accessible the specimen

TABLE V.

*Procedure for Examining Inactivated Human Sera.**

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Patient's serum (inactivated) 0.2 cc. Active negative serum 0.1 cc.† Antigen 0.1 " 0.9 per cent saline solution 0.9 cc.	First incubation for 30 min. in water bath or 1 hr. in air thermostat.	Anti-human amboceptor 1 unit in 0.1 cc. of 10 per cent human corpuscular suspension, 0.1 cc. Total volume 1.5 cc. Contents are well mixed.	Second incubation same as first except that tubes are shaken three times during incubation.	Reading of results within 30 min. after removal of tubes from incubator.
Control tube (back row).	Patient's serum (inactivated) 0.2 cc. Active negative serum 0.1 cc.† Antigen omitted. 0.9 per cent saline solution 1 cc.				

* This applies also to unheated sera which have too little complement from the beginning or have lost complement on standing. Old sera are often anticomplementary and for such specimens only 0.1 cc. is indicated.

† A second complete test of this serum should accompany the test of the serum to which it is added as complement.

TABLE VI.

Procedure for Examining Cerebrospinal Fluid.

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Cerebrospinal fluid 0.2 cc.* Active negative serum 0.1 cc.† Antigen 0.1 " 0.9 per cent saline solution 0.9 cc.	First incubation same as in Tables IV and V.	Anti-human amboceptor 1 unit in 0.1 cc. of 10 per cent human corpuscular suspension, 0.1 cc. Total volume 1.5 cc. Contents are mixed by shaking.	Second incubation same as first except that contents of tubes are shaken three times during incubation period.	Reading of results within 30 min. after removal of tubes from incubator.
Control tube (back row).	Cerebrospinal fluid 0.2 cc. Active negative serum 0.1 cc.† Antigen omitted. 0.9 per cent saline solution 1 cc.				

* Graduated quantities of from 0.2 to 0.5 cc. may be used in certain cases, the amount of saline solution being so adjusted as to make the total volume 1.3 cc.

† A second complete test of this serum should accompany the test of the serum to which it is added as complement.

may always be acetated, since the presence of the acetate does not interfere with the test (Rhamy).

The acetate may be used as preservative for the serum in either of two ways. A sterile solution, containing 0.9 per cent sodium chloride and 10 per cent sodium acetate, may be mixed in equal parts with the serum after separation from the clot; or 0.5 cc. of a sterile concentrated acetate solution (50 per cent) in 0.9 per cent saline solution may be placed in a graduated centrifuge tube and the blood drawn from the patient directly into the tube up to the 5 cc. mark, the whole being then shaken thoroughly. Coagulation takes place as usual, and the clot is just as firm as in a control tube in which 0.5 cc. of saline solution is added to the same amount of blood. There is no disturbing effect from the presence of the acetate in the serum, either when it is fresh, or when it has stood for several days. The serum acetate mixture may be kept in the refrigerator, where it will retain complement activity longer than at room temperature; in the latter, activity is preserved for several days. Once the acetate serum has become inactive by long standing, it can still be tested by adding fresh human complement.

The method of mixing clear serum with the saline acetate solution is much to be preferred to the direct mixing of the blood and concentrated acetate solution.

Preparation of Reagents.

Although the details with regard to the reagents used in conducting the present test are essentially the same as those described in earlier publications[6, 16] dealing with the anti-human heterohemolytic system (the use of guinea pig complement with anti-human amboceptor and human corpuscles), it nevertheless seems desirable to summarize them briefly here. Antigen, anti-human hemolytic amboceptor, and a suspension of human blood corpuscles are the reagents required to test the patient's serum.

Antigen.—The preparation, titration for its antigenic properties, and preservation of the antigen are described in other publications dealing with the subject in minute detail.[6] Suffice it to say that the acetone-insoluble fraction of tissue

[16] Noguchi and Bronfenbrenner, Biochemical studies on so-called syphilis antigen, *J. Exp. Med.*, 1911, xiii, 43.

lipoids is recommended, 0.1 cc. of a 1: 10 emulsion with 3 per cent methyl alcohol solution (stock) and 0.9 per cent saline solution being used for each test. No preparation which hemolyzes in 0.4 cc. or interferes with complement in the same dose should be used. The antigen should be effective in doses of 0.02 cc. of the emulsion. In the actual test at least five antigenic units are employed in order that no positive reaction may be overlooked. The methyl alcohol stock solution remains active indefinitely at room temperature, and a saline emulsion of 1: 10 strength may be made up at any time. The latter, when kept in a refrigerator, remains unchanged for several days, although it is best to make a fresh emulsion on the day that tests are to be made.

Amboceptor.—The production of the anti-human amboceptor is one of the most important parts of the present method. Rabbits, when immunized with thoroughly washed human corpuscles in sufficiently large quantities, yield a powerful serum, which is able to produce complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in doses of from 0.01 to 0.005 cc. This point will be discussed at greater length later. The usual amount of this reagent used in the test is 0.1 cc., which is so made as to represent one hemolytic unit in the presence of 0.1 cc. of fresh human serum and against 1 cc. of the 1 per cent corpuscular suspension, hemolysis becoming complete within 20 to 30 minutes at 37°C. in a water bath or 1 hour in an air thermostat.

Corpuscular Suspension.—0.1 cc. of a 10 per cent suspension is best suited for the purposes of the test. The suspension is prepared as follows: 1 part of the washed corpuscles from any individual (it is convenient to use corpuscles obtained from one of the patients being bled for test serum) is mixed with 9 parts of 0.9 per cent saline solution. A few cubic centimeters of blood are drawn into a centrifuge tube containing an equal volume of sodium citrate solution (2 per cent in 0.9 per cent saline solution) and the mixture is repeatedly centrifuged with changes of saline solution until there is no more serum in the supernatant fluid. The final sediment of corpuscles is suspended in a quantity of saline solution equal to the original quantity of the blood. The corpuscular suspension thus prepared should be used when fresh and when not in use should be kept in a refrigerator, where it can be preserved for a period of about 72 hours. A suspension older than 72 hours should not be used, however, because the use of corpuscles with diminished resistance to hemolysis may cause the masking of a possible weak positive reaction.

Utilization of the Patient's Corpuscles.—For any well equipped laboratory the preparation of a corpuscular suspension of a definite concentration by the above method offers no difficulty. There may be occasions, however, for example on board ship or in field hospitals, when no centrifuge is available. Under these conditions the preparation of a uniform corpuscular suspension for an entire set of tests is not possible, and to meet this sort of emergency the utilization of the patient's own corpuscles for testing his serum is recommended. As has already been emphasized, the serum of the patient to be examined must be perfectly fresh and the corpuscles which can be liberated by gently stirring the clot are correspondingly fresh and can be used as the indicator of hemolysis. The fol-

lowing technique gives the best result: 0.4 cc. of the fresh clear serum from the tube containing the coagulated blood is measured out and put into one of the two tubes used for the test. 2 cc. of 0.9 per cent saline solution are added, making a total volume of 2.4 cc. After putting the remaining clear serum into a tube for future use, the clot is gently shaken, by means of a medium sized pipette, to liberate enough corpuscles to tinge the serum dilution to the desired color value (suspension). The standard of suspension aimed at is 1 per cent of corpuscles, and after a certain amount of practice no difficulty is experienced in detecting the difference between a 1 per cent and a 1.5 per cent suspension by this means. A 1 per cent suspension is an opaque fluid with a yellowish red hue. As the concentration of the suspension increases, the red becomes more predominant over the yellow tint. Having made a mixture of 0.4 cc. of the fresh serum and approximately a 1 per cent corpuscular suspension in a total of 2.4 cc., one divides this quantity into two equal portions by measuring out 1.2 cc. into the second tube of the set. One of the two tubes receives the antigen 0.1 cc., and the other receives the saline solution 0.1 cc., and serves as the control without the antigen. Both are incubated for 30 minutes in a water bath or 1 hour in an air incubator. 0.1 cc. of the anti-human amboceptor, representing one hemolytic unit, is then added to both tubes, and after thorough mixing the tubes are again incubated, as in all other procedures.

When the test is done in this way, the corpuscles are, of course, present from the beginning, instead of being added after the first incubation simultaneously with the amboceptor, as in procedures in which corpuscles from other patients are used. But in any procedure the corpuscles may be introduced from the beginning and the final result remain the same.

Tschernogubow[8] once proposed the use of a suspension of the patient's blood directly diluted in saline solution in a ratio of 1 drop to 1 cc. of saline. This was to serve as the source of complement, corpuscles, and, if present, the fixing substance. By careful scrutiny, however, one soon discovers the enormous disproportion among the various elements concerned. For example, the amount of serum probably present in 1 drop of blood, which would perhaps be 0.07 cc., would be approximately 0.035 cc. at most (about one-sixth the amount used in the writer's system), while the corpuscular suspension approaches 7 per cent (seven times the concentration in the writer's system). The amount of syphilitic antibodies present would be too small to make possible the detection of a weak positive reaction, and the minuteness of complement present in such a mixture precludes any practical possibility. Even an enormous amount of the amboceptor fails to complete hemolysis of such a concentrated

suspension of the corpuscles. Moreover, the mixture forms a loose gelatinous fibrin, involving the whole contents, making it impossible to stir by shaking. When the first fibrin is removed, a second may form on further standing. Such a method cannot be used, and Tschernogubow himself soon abandoned it.

Emery[9] employs active serum with the cholesterolized alcoholic extract of heart muscle, disregarding the possibility of obtaining a false positive fixation with certain non-syphilitic sera. The amount of the patient's serum is minute, but the concentration of the corpuscles (20 per cent of the firmly packed sediment after centrifugation) is almost six times that used in the method being proposed. There is no economic gain in Emery's method, therefore, since the amount of amboceptor required is no less than that used in the proposed method. Moreover, the manipulation of minute quantities of various factors by Wright's capillary technique requires a considerable degree of skill as compared with the ease with which regular graduated pipettes can be handled.

Butler and Landon[10] and Myer[11] inactivate the patient's serum before the test and add fresh negative human serum from a non-syphilitic individual as complement. They recommend the use of sensitized human corpuscles and the acetone-insoluble tissue lipoids (Noguchi). Their method is decidedly more rational than Emery's and seems to have given satisfactory results in 300 cases so far reported.[16] They also employ Wright's capillary technique.

Thompson[12] uses fresh human serum, with preliminary titration of each specimen for its complement activity. This procedure seems to be unessential, since the majority of specimens already contain enough complement. The amount of corpuscles used is 0.2 cc. of a 2 per cent suspension. A stronger concentration would give a more distinct reaction.

Results of Practical Application of the Test.

The writer has been able, up to the present time, to examine 1,331 specimens of blood and 52 cerebrospinal fluids.[17] Of 1,118 specimens of sera from these sources 517 gave a positive and 601 a negative

[17] I wish to express my appreciation to the members of the staffs of several hospitals through whose cooperation this work was made possible.

reaction, the results conforming to those reported by the serological departments of the various hospitals. Of 132 specimens from psychiatric cases, 54 were from general paralysis cases, and all except 2 gave a strongly positive reaction. Among other psychoses, including 75 cases of dementia præcox, 3 of alcoholic psychosis, 3 of imbecility, 3 of senile psychosis, 6 of arteriosclerosis, 1 of manic-depressive insanity, and 1 paranoic condition, there were only 2 positive reactions, these occurring among the dementia præcox cases. The reactions with 81 inactivated sera agreed with those obtained by others with the same material. 20 cerebrospinal fluids from cases of general paralysis gave a strongly positive reaction, while 32 specimens from other non-syphilitic cases showed a negative reaction. The statement will perhaps bear repeating that of 1,250 fresh human sera complement was deficient in 93 specimens, which had to be examined either by means of additional amboceptor or by supplying active human complement from fresh negative sera. This special adjustment with fresh sera is one which demands particular attention on the part of serologists adopting the method.

Quantitative Consideration of the Complement Fixation Test.

In an ideal method for the serum diagnosis of syphilis every ingredient should be separately controllable by accurate titration. This is possible with the anti-human heterohemolytic system (Noguchi), in which the amount of guinea pig complement is accurately measured and added to a definite quantity of serum, whose native complement plays no part in the reaction, or has been removed by inactivation, and in which the antigen, anti-human amboceptor, and corpuscles are equally definite, no factor being present which can give rise to a quantitative disturbance. The homohemolytic system, upon critical examination, will be seen also to be capable of equal accuracy. The possible sources of error and the methods of adjusting them are discussed below.

The Frequency and Extent to Which a Positive Reaction May Be Masked by an Excess of Complement.—Only 2 per cent of several hundred specimens examined in this study were found to contain an extra unit of complement activity. None contained three units.

An excess of complementary activity may cause a positive serum to give a weakly positive or even a negative reaction, but only when the so called antibody content of the serum is less than one fixing unit. For example, a specimen containing half of an antibody unit in the presence of two complement units may give a negative result. No change in the reaction can occur, however, from an excess of less than two complement units in the presence of $1\frac{1}{2}$ units of syphilitic antibody. Error from this source is therefore extremely rare, and the reaction cannot be completely negative when the specimen contains more than one antibody unit.

TABLE VII.

Relation between Various Quantities of Human Complement and of Antibody.

Syphilitic serum (active).		Fresh negative human serum as complement.						
Amount.	No. of units of antibody.	0.1 cc.	0.125 cc.	0.15 cc.	0.2 cc.	0.25 cc.	0.3 cc.	0.4 cc.
cc.								
0.008	0.5	+++	+++	++	++	+	+	−
0.016	1.0	++++	++++	++++	++++	+++	+++	+
0.024	1.5	++++	++++	++++	++++	++++	+++	++
0.032	2.0	++++	++++	++++	++++	++++	++++	+++
0.04	2.5	++++	++++	++++	++++	++++	++++	++++
0.048	3.0	++++	++++	++++	++++	++++	++++	++++
0.064	4.0	++++	++++	++++	++++	++++	++++	++++

Table VII records experiments in which the relation between various quantities of human complement and of antibody was determined. The amount of anti-human amboceptor used was that which produced complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in the presence of 0.1 cc. of fresh human serum (one complement unit) within 20 to 30 minutes at 37°C. (water bath). Table VII shows that the addition of quantities of the human complement ranging from 0.1 to 0.2 cc. did not materially change the ++++ reaction when combined with 0.016 cc. of syphilitic serum. The reactions became somewhat weaker, however, when 0.15 and 0.2 cc. of the complement were used with 0.008 cc. of the serum. When the amount of the syphilitic serum was increased to 0.024 to 0.032 cc. or more, the reaction was ++++ against 0.3 cc. of the complement, and

0.04 cc. of serum prevented hemolysis in the presence of 0.4 cc. of complement. The reduction in the degree of positive reaction caused by the variations of 0.1 to 0.2 cc. of the human complement is insignificant and has no serious effect upon the ultimate result when the serum contains more than $1\frac{1}{2}$ antibody units. The apparent disproportion of complement in the routine amount of 0.2 cc. of the fresh serum is well balanced by the antibody content, which is four to five times that of the same serum inactivated. For that reason only 0.1 cc. of human serum is added to 0.2 cc. of inactivated serum. This proportion is similar to that of the guinea pig complement and inactivated serum used in the Wassermann system and in the anti-human heterocomplement system (Noguchi). But the addition of 0.2 cc. of the human complement does not mask a strongly positive reaction (++++).

The Possibility of Interference by Negative Serum in Complement Fixation in the Homohemolytic System.—The use in the homohemolytic system of fresh negative serum as complement when there is no complement or too little in the specimen to be examined, while entirely analogous to the use of fresh guinea pig serum as complement for an inactivated patient's serum, may rouse apprehension as to the possibility of reduction in the degree of fixation by this comparatively large amount of human complement, owing to its indifferent serum constituents. The experiments recorded in Table VIII, in which human and guinea pig complement were studied in parallel series, show that the addition of an inactivated negative serum to a syphilitic serum does not cause any so called complementoid blocking of the complement fixation of any significance, and that no error can result from this source.

All the other experiments of this sort with syphilitic sera gave similar results. It was found, however, that a syphilitic serum containing less than one-half an antibody unit caused less inhibition in the tubes to which more than 0.3 cc. of the inactivated negative human serum had been added, but never completely masked the reaction. There was no appreciable difference in the tubes containing 0.1 or 0.2 cc. of the inactivated serum and that containing none. The addition, however, of inactivated guinea pig serum (56°C.) to a syphilitic serum produces marked weakening of the fixation reaction, and even com-

TABLE VIII.

Effect of Inactivated Negative Human Serum upon the Complement Fixation.

	With human complement.	Results.	With guinea pig complement.	Results.
With inactivated syphilitic serum.	Syphilitic serum (56°C.) containing 1 antibody unit 0.2 cc. Human complement (active serum) 0.1 " The same + negative human serum (56°C.) 0.1 " The same + negative human serum (56°C.) 0.2 " The same + negative human serum 56°C.) 0.3 " The same + negative human serum (56°C.) 0.4 "	All gave complete fixation, regardless of the addition of the inactivated negative serum.	Syphilitic serum (56°C.) containing 1 antibody unit 0.2 cc. Guinea pig complement 40 per cent 0.1 " The same + negative human serum (56°C.) 0.1 " The same + negative human serum (56°C.) 0.2 " The same + negative human serum (56°C.) 0.3 " The same + negative human serum (56°C.) 0.4 "	All gave complete fixation, no interference being observed from the addition of the inactivated negative human serum.
With active syphilitic serum.	Active syphilitic serum, containing 4 antibody units 0.2 cc. The same + negative human serum (56°C.) 0.1 "	Complete fixation in all.		

TABLE VIII—*Concluded.*

	With human complement.	Results.	With guinea pig complement.	Results.
With active syphilitic serum.	The same + negative human serum (56°C.) 0.2 cc. The same + negative human serum (56°C.) 0.3 " The same + negative human serum (56°C.) 0.4 "	Complete fixation in all.		

plete blocking when more than 0.3 cc. of that serum is added to one syphilitic antibody unit. This confirms earlier observations.[18]

Result of the Presence of an Excess of Amboceptor in the Complement Fixation Test.—The disturbance resulting from an excess of amboceptor in any complement fixation test has been repeatedly pointed out and is recognized by impartial workers as inherent in the anti-sheep hemolytic system. Removal by absorption of the natural anti-sheep amboceptor from each specimen of serum prior to test has been advocated, but for obvious reasons is impracticable when several dozen specimens must be examined at one time.

In Table IX are recorded the results obtained in parallel series of tests,[19] the anti-sheep system with guinea pig complement being used in one series, and the anti-human homocomplement system in the other. These experiments confirm earlier observations[20] that a

[18] Noguchi and Bronfenbrenner, The interference of inactive serum and egg-white in the phenomenon of complement fixation, *J. Exp. Med.*, 1911, xiii, 92.

[19] These tests were carried out by Major Felix R. Hill, Captain George L. Schadt, and Lieutenant Ralph R. Simmons in this laboratory.

[20] Noguchi, Die quantitative Seite der Serodiagnostik der Syphilis, *Z. Immunitätsforsch., Orig.*, 1911, ix, 715.

positive serum containing 1 antibody unit can become completely negative when 4 amboceptor units are used, or 3 antibody units with 20 amboceptor units. Table IX shows that 1 antibody unit is made negative by 6 amboceptor units and 3 antibody units by 10 amboceptor units with the Wassermann system, while with the anti-human homocomplement system nearly twice the amount of amboceptor is required to produce the same effect.

The phenomenon just described assumes practical importance in any system in which are used foreign blood corpuscles for which human serum normally contains varying amounts of natural hemolytic amboceptor, in which case there is a possibility of an excess of amboceptor in the test. It does not occur in an anti-human hemolytic

TABLE IX.

Reversion of Reaction through Excess of Amboceptor.

No. of units of amboceptor.	Syphilitic antibody 1 unit.		Syphilitic antibody 3 units.		Syphilitic antibody 10 units.	
	Wassermann anti-sheep system.	Anti-human homocomplement system.	Wassermann anti-sheep system.	Anti-human homocomplement system.	Wassermann anti-sheep system.	Anti-human homocomplement system.
1	++++	++++	++++	++++	++++	++++
2	++++	++++	++++	++++	++++	++++
3	++	++++	+++	++++	++++	++++
6	--	+++	+	++++	++++	++++
10	−	−	−	++	++++	++++
20	−	−	−	−	+++	+++
40	−	−	−	−	+	+

TABLE X.

Titration of Natural Anti-Sheep Amboceptor in Human Serum.

Sera.	No. of specimens examined.	No. of units of anti-sheep amboceptor in 0.2 cc. of human serum (56°C.), titrated with guinea pig complement.										
		None.	< 1	1	2	3	4	5	6	7	8	>10
Syphilitic sera	190	19	21	42	21	25	26	17	8	4	3	4
Non-syphilitic sera	111	3	15	25	31	10	12	8	5	1		1
Normal sera	25	1	3	5	4	3	1	2	2	3	1	
Total	326	23	39	72	56	38	39	27	15	8	4	5

system, whether guinea pig complement or human complement is used, and especially when the patient's own corpuscles are employed for each specimen. Data which have been collected concerning the amount of natural anti-sheep amboceptor in 326 specimens of human serum are given in Table X.

Tables IX and X explain the possibility of a reduced or reversed reaction with certain syphilitic sera in the anti-sheep system. The frequency and extent of error from this source are much greater than those due to slight variations in complement content, which are readily amenable to quantitative adjustment.

Quantitative Estimation of the So Called Syphilitic Antibody.—If necessary, any strongly positive serum can be titrated by the homo-complement system, the procedure being the same as that recommended for the anti-human heterocomplement system; namely, that of keeping all the other ingredients constant and determining the smallest quantity of the serum which will give complete fixation. For this purpose the serum may be inactivated before titration. To a number of tubes containing 0.1 cc. of the fresh human complement (previously titrated) and the standard antigen (usually contained in 0.1 cc. of a suitable dilution) are added varying amounts (ranging from 0.01 to 0.2 cc.) of the positive human serum (inactivated) to be titrated, and the mixture is made up to 1.3 cc. in each tube. After an incubation at 37°C. for 30 minutes in the water bath or 1 hour in an air incubator, the corpuscle suspension (0.1 cc.) and one unit of amboceptor (0.1 cc.) are added, and a second incubation follows. The smallest quantity of serum which produces complete fixation is taken as one antibody unit.

SUMMARY.

The elimination of the foreign complement and corpuscles from the test for the serodiagnosis of syphilis has been attempted, and the results so far obtained are very satisfactory. Instead of using guinea pig complement, fresh human serum was utilized for the source of complement for the production of hemolysis upon the human corpuscles in the presence of an adequate amount of the specific anti-human amboceptor (prepared in rabbits). Usually 0.1 cc. of fresh

human serum contains enough complement to hemolyze 1 cc. of a 1 per cent suspension of human corpuscles, but the amount of anti-human amboceptor required in this combination is about five to seven times that necessary when guinea pig complement (0.04 cc.) is used. It has also been shown that when a given human serum contains insufficient complement, an adequate quantity (0.1 cc. is usually enough) of another fresh negative serum may be added to supplement it. However, one rarely encounters this group of sera. Inactivated human sera can also be examined by utilizing human complement from another source (the serum must be fresh, active, and negative).

The only drawback to the present method is the comparatively large amount of anti-human hemolytic amboceptor required. It is estimated that 30 to 40 cc. of the anti-human hemolytic immune serum, from one rabbit, of high potency—say 0.005 cc.—would be enough to examine about 3,000 to 4,000 cases (0.01 cc. for each case), whereas if guinea pig complement were used the same amount would cover about 15,000 tests (0.002 cc. for each case). In a large hospital or in the Army there should be no difficulty in preparing any amount of the anti-human hemolytic amboceptor. For example, material for 100,000 tests could be prepared within 1 month in less than 100 rabbits. The amboceptor serum can be used in the fluid state, or, if the titer is high, impregnated into filter papers.

Special attention should be called to the fact that to obtain a powerful anti-human hemolytic amboceptor five to six intraperitoneal injections of corpuscles, thoroughly washed (until there is no trace of serum in the supernatant fluid), in doses of 5, 7, 10, 10, 10, and possibly another 10 cc. of the concentrated suspension (restored to the original volume of the blood) are required.[21] The bleeding may be done by the 9th or 10th day. The animals may be kept after bleeding for the production of more amboceptor by subsequent injections of the washed corpuscles.

Finally, it should be emphasized that only the acetone-insoluble fraction of tissue lipoids of required standards (Noguchi) should be used when utilizing the human complement in the fixation test.

[21] Intravenous injections of .3, 3, 4, and 4 cc. of the corpuscular suspension every 4 or 5 days also give excellent results.

AN EXPERIMENTAL STUDY OF VACCINATION AGAINST BACILLI DYSENTERIÆ.

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The occurrence of bacillary dysentery in the armies of Europe, the finding of great numbers of cases of this infection in the tropics, where the amebic form has been regarded as the prevailing type, and the presence of the disease endemically in the United States have stimulated this series of investigations.

Attempts have been made at immunization against bacillary dysentery by means of cultures of the dysentery bacilli from the time of their discovery by Shiga up to the present, yet no definite method has been found which made vaccination or active immunization practicable.

An analysis of previous efforts reveals the peculiarities of the *B. dysenteriæ* group of organisms. Shiga,[1] in 1898, injected himself subcutaneously with one-twelfth of a killed agar slant culture of *B. dysenteriæ* (Shiga). The local reaction was of such intensity that eventually incision was made to evacuate the purulent exudate and to help the absorption of the board-like edema. However, after 10 days agglutinins were demonstrable in his blood. So also Kruse,[2] who injected himself with 1 cc. of a killed bouillon culture, developed a severe local reaction persisting for a week. After 8 days the agglutinin titer of his serum was 1: 800. Rosenthal,[3] using a similar antigen upon himself, also developed a marked local and general reaction, but no agglutinins.

These personal experiments were supplemented by animal experiments by others, and the conclusions reached were that simple suspensions of the killed bacilli in saline solution, or killed bouillon cultures, while too toxic for practical use, do nevertheless give rise on inoculation to antibody production.

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[1] Shiga, K., *Centr. Bakteriol., 1te Abt.*, 1898, xxiv, 817, 870, 913.

[2] Kruse, W., *Deutsch. med. Woch.*, 1900, xxvi, 637; 1901, xxvii, 386; 1903, xxix, 49.

[3] Rosenthal, *Centr. Bakteriol., 1te Abt., Ref.*, 1905, xxxvi, 23.

Attention was next concentrated upon the addition of immune serum to the bacteria, either to sensitize the antigen or to neutralize its toxicity. Shiga,[4] proceeding on the latter basis, injected killed cultures and immune serum simultaneously for the first injection, following it in 3 to 4 days by a larger dose of the bacilli without serum. He vaccinated 10,000 Japanese in this way and determined that a definite protection was afforded, since the mortality was reduced. The general results, however, were unsatisfactory, as the immunity endured but 3 to 4 weeks. Subsequently Shiga varied the dosage by giving antiserum with the second dose, but although the local and systemic reactions were mitigated, the procedure failed to produce a lasting immunity.

Vaillard and Dopter[5] and Dopter[6] employed the Besredka method of sensitizing vaccines and obtained what they regarded as encouraging results, but Lüdke[7] was unable to detect any advantage in the sensitized vaccine. Lüdke's conclusions are that sensitization not only removes the toxicity of the dysentery bacillus but also destroys its antigenic function. Lucas and Amoss[8] employed a vaccine consisting of Flexner organisms in combination with immune serum, but on account of the few instances studied no definite conclusions can be drawn. Immune serum alone has been used by Shiga, Dopter, and others as a prophylactic measure, but it is generally conceded that the passive immunity endures usually only about 10 days. Recently Gibson[9] removed by absorption the so called antibacterial principles of immune serum, leaving the supposed anti-endotoxic elements. The absorbed serum was injected with the killed cultures, and Gibson states that the severe local reaction is abolished and an immunity conferred. It will be shown later that Gibson's serum possesses no advantage over unmodified immune serum.

While the foregoing observations were being made another series of tests revealed that immunization is possible after injection of dysentery bacilli in either changed or unchanged form. Gay[10] ascertained that repeated injections of the organisms in guinea pigs produces an active immunity. Lüdke[11] showed that rabbits, after repeated intravenous injections of cultures, develop immune serum of high titer, possessing strong bactericidal powers, agglutinins, complement-fixing antibodies, and precipitins. A similar result follows subcutaneous injection. It is important to note that the agglutinin titer corresponded to the protective value of a given serum. Immunity, though not of such a degree, could also be

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[4] Shiga, K., *Deutsch. med. Woch.*, 1903, xxix, 327.
[5] Vaillard, L., and Dopter, C., *Ann. Inst. Pasteur*, 1903, xvii, 463.
[6] Dopter, C., *Ann. Inst. Pasteur*, 1909, xxiii, 677.
[7] Lüdke, H., Die Bazillenruhr, Jena, 1911, 128.
[8] Lucas, W. P., and Amoss, H. L., *J. Exp. Med.*, 1911, xiii, 486.
[9] Gibson, H. G., *J. Roy. Army Med. Corps*, 1917, xxviii, 615.
[10] Gay, F. P., *Univ. Penn. Med. Bull.*, 1902–03, xv, 307.
[11] Lüdke, H., *Centr. Bakteriol., Ite Abt., Orig.*, 1905, xxxix, 512, 649.

secured by using autolyzed products of the dysentery bacilli (Neisser and Shiga[12]). The dried powder of dysentery bacilli, suspended in saline solution and injected intravenously into rabbits, has also been used. It produces agglutinins, though not to a great degree. The bacilli digested with pepsin or trypsin have also been tested and an actively bactericidal immune serum has been obtained with them.

All these methods demonstrate the possibility of developing immunity through the injection of the proteins, in some form, of the dysentery bacilli. An analysis of the results will show the advantage of using as antigen bacilli as nearly in their natural state as possible, and such a method conforms with the general principles of active immunization as laid down by Theobald Smith.[13]

With the results of the earlier work before us, keeping in mind the high toxicity of the dysentery bacilli for human beings and considering the importance of employing them as little changed by artificial manipulation as possible, we undertook the following experiments. The objects of the experiments were the finding of a suitable medium for suspending the bacilli, the diminution of their toxic effects, and the retention of their immunizing values.

Several kinds of vaccine of the dysentery bacilli were prepared. It is generally conceded now that the dysentery bacilli may be divided into two main groups, the Shiga and the Flexner. Organisms of either group may produce similar intestinal lesions and clinical symptoms. They are, however, distinguishable by their power to ferment certain carbohydrates and alcohols, and by the fact that the Shiga bacillus yields, together with an endotoxin, what appears to be an exotoxin, while the Flexner bacillus does not. The Shiga group is homogeneous, the Flexner group heterogeneous. In the latter is included the representative type, isolated by Flexner[14] in the Philippine Islands in 1900, the Hiss Y,[15] and the Strong[16] types. The organisms of the Flexner group possess common antibodies: cross-agglutination, as well as cross-protection, is definite. The organisms of the Shiga group are, however, distinguished serologically from those of the Flexner: cross-agglutination and cross-protection do not occur, unless exceptionally, in a small degree.

[12] Neisser, M., and Shiga, K., *Deutsch. med. Woch.*, 1903, xxix, 61.

[13] Smith, Theobald, *J. Am. Med. Assn.*, 1913, lx, 1591.

[14] Flexner, S., *Philadelphia Med. J.*, 1900, vi, 414.

[15] Hiss, P. H., Jr., and Russell, F. F., *Med. News*, 1903, lxxxii, 289.

[16] Strong, R. P., and Musgrave, W. E., Report of the etiology of the dysenteries of Manila, *Rep. Surg.-Gen. Army*, Washington, 1900, 251.

Hence, in the following experiments the vaccines were made polyvalent, in order to include the antigenic factors of the two groups of organisms. As a representative of the Shiga group a known culture was selected, of definite toxicity. The minimum lethal dose of its toxin fluctuated between 0.05 and 0.1 cc. for a 1,500 to 1,800 gm. rabbit. As a representative of the Flexner group the original Flexner bacillus (the Flexner-Harris, or Philippine strain) was chosen, as well as the Hiss Y, the two organisms present in epidemic and sporadic cases of dysentery. The three organisms were present in the vaccines in equal proportions with reference to their grouping so that an injection would include one-half the total of Shiga organisms, one-quarter of Flexner bacilli, and one-quarter of Y bacilli.

Saline Dysentery Bacilli Vaccine.

The usual saline suspensions of the bacilli were first studied in order to control the antigenic properties of the organism and to confirm previous observations. To this end the following experiment was performed.

Experiment A.—There were employed suspensions of Shiga and Flexner bacilli in isotonic saline solution, of which each cubic centimeter contained 4 billion organisms, killed by heating at 60°C. for 30 minutes, preserved by means of 0.35 per cent tricresol.

Three rabbits, Nos. 1, 2, and 3, were injected subcutaneously with this vaccine. The first dose was 0.25 cc., or 1 billion bacteria, the second dose was 0.5 cc., or 2 billion, and the third was 0.75 cc., or 3 billion. The injections were given at 5 day intervals, under the shaven skin of the abdomen. After 24 hours the three rabbits developed a severe edematous swelling which, as a rule, extended over an area 6 cm. in diameter. There were tenderness and redness, and eventually firm nodules formed. Absorption was slow; usually a week elapsed before the nodule receded to a small, firm, papular mass. Rabbits 1 and 2 succumbed to the third injection.

Agglutination tests were made from time to time, the macroscopic method being employed, and the tubes being kept at 55°C. for 4 to 5 hours.[17] Rabbit 3 showed

[17] It was determined that this method gave much more definite reactions than the old method of incubating for 2 hours at 37°C. and then keeping over night in the ice box. The specificity of the agglutination was not affected by the heat. It was subsequently found that these tests could be left at 55°C. over night with good results.

no agglutination 5 days after the first injection. 5 days after the second injection the serum agglutinated Flexner bacilli in 1:100, but not Shiga bacilli. 7 days after the third injection Flexner and Shiga bacilli were agglutinated in 1:100, and 1 month after the injection the Flexner agglutination was 1:100 and the Shiga negative. Rabbit 2 showed Flexner agglutination 1:100 5 days after the first injection and 1:800 a similar period after the second. This rabbit died 24 hours after the third injection. Rabbit 1 also showed agglutination of 1:100 for Flexner bacilli and 1:50 for Shiga 5 days after the first injection, and 1:200 for Flexner and 1:100 for Shiga 7 days after the third injection. At this time the rabbit died.

The surviving rabbit was injected 1 month after the last vaccination with ten lethal doses of a Shiga culture intravenously. Two controls, similarly injected, died in 18 and 24 hours respectively. The vaccinated animal survived.

From this experiment we may conclude that saline vaccines are too toxic for use: two of the three rabbits succumbed to the injections. At the same time they are capable of developing considerable agglutinins—the agglutination titer was high in all three rabbits. They also afford protection to many times the fatal dose of the Shiga bacillus. With respect to the toxicity, the antigenic property, and the protective effect, the previous observations have been confirmed.

Vaccines Suspended in Chemical Solutions Other than Saline.

A chemical solution was first sought which would approach as closely as possible the concentration and acidity of tissue fluids. For this purpose Henderson's phosphate solution[18] was employed. It contains a 1 per cent concentration of monosodium phosphate 1 part, and disodium phosphate 9 parts.

Experiment B.—The bacilli in the vaccine were prepared in the usual manner and added to the phosphate solution. Three rabbits (Rabbits 4, 5, and 6) were injected in the same manner and with the same dosage as in Experiment A. In none of them was there any local reaction. Rabbits 4 and 5 died after developing paralysis of both anterior and posterior extremities. In none of this series were agglutinins demonstrable. Rabbit 6 alone survived the three injections and was injected 1 month after the last injection with ten lethal doses of a Shiga culture intravenously. The animal succumbed, together with the controls.

[18] Henderson, L. J., *J. Biol. Chem.*, 1909–10, vii, 29.

This method was unsuccessful, since neither agglutinins nor protection developed, and although the local toxicity was diminished, the systemic toxicity was apparent.

The next trial was based on the possibility that the irritation following an injection of the vaccine might be due to acid production in the tissues. Hence a new vaccine was prepared, an alkaline medium being used, consisting of lime-water (aqueous solution of calcium hydroxide), for suspending the killed bacilli.

Experiment C.—Three rabbits (Nos. 7, 8, and 9) were injected in the usual manner (see Experiment A). Rabbit 8 died after the first injection, of typical dysentery intoxication. No local reaction was seen in any of this series. No agglutinins were produced. The two surviving rabbits showed no protection against fatal doses of Shiga and Flexner type cultures.

With alkaline suspensions, then, no agglutinins were produced, nor was protection afforded. Although there was no local reaction, the systemic toxicity was not diminished.

From these experiments we may conclude that the presence of certain chemicals in a vaccine may eliminate its local irritating effect but at the same time will remove its antigenic power and simultaneously increase its systemic toxic effects.

Immune Serum Alone and with Vaccines.

As a control of the experiments to follow, one series of rabbits was injected with polyvalent antidysenteric serum alone.

Experiment D.—Rabbit 10 was injected with 1 cc. of polyvalent antidysenteric serum subcutaneously three times at 5 day intervals. There was no local reaction. No agglutinins were demonstrable. 1 month after the last injection there was no protection against ten lethal doses of a Shiga culture. Repetition of this experiment afforded similar results.

The immune serum alone is incapable of producing agglutinins in dilutions of 1:25 to 1:50, nor is there protection at the end of a month. Hence, whatever results in agglutinin production, protection, or local reaction occurred in the following series are to be ascribed to the bacterial vaccine and not to the immune serum. The uncertainty of duration of the effect of the prophylactic injection of immune serum was incidentally demonstrated.

The next experiment was undertaken to show the effect of the ordinary saline suspension vaccines combined with the use of polyvalent antidysenteric serum.

Experiment E.—The dosage of the vaccine and the method of injection were similar to those mentioned in Experiment A, except that with each injection 1 cc. of the immune serum was given subcutaneously.

Three rabbits (Nos. 11, 12, and 13) were tested for the presence of normal agglutinins for the dysentery bacilli on Oct. 4, 1917, a procedure which was part of the routine of all experiments. None having been detected, the animals were injected shortly afterwards, as already indicated. In none of the rabbits was there any local reaction. Agglutinins were produced in all of them. As a rule the serums agglutinated Flexner bacilli in 1: 50, but not Shiga bacilli, 5 days after the first injection. 5 days after the second injection the titer rose, as a rule, to 1: 200 for Flexner and 1: 100 for Shiga cultures. 7 days after the last injection the titer was 1: 200 for both type cultures. 1 month after the last injection Rabbit 11 showed 1: 50 Flexner and Shiga agglutinations, Rabbit 12, 1: 200 Flexner and Shiga agglutinations, and Rabbit 13 died of an intercurrent infection (coccidiosis) before the end of that period. Rabbit 11 showed resistance to four lethal doses of a Flexner culture, although controls died in 18 and 24 hours respectively, but Rabbit 12 succumbed to ten lethal doses of a Shiga culture.[19]

The deduction from this series is that immune serum exerts a neutralizing effect upon the toxicity of the cultures, permitting the production of agglutinins in an appreciable amount and giving rise to partial protection, enduring at least a month after the last injection.

Gibson,[2] to whom reference has already been made, attempted the removal of the antibacterial constituents from immune serum and the retention of the antiendotoxic substances. The method employed was the complete absorption by means of bacterial suspension of all agglutinins and other antibacterial antibodies present in polyvalent antidysenteric serum. This modified serum was injected with killed cultures of dysentery bacilli. In the next experiment an attempt was made to investigate the merits of this form of vaccination.

[19] Four lethal doses of a Flexner culture were used in these tests for protection for the following reason. Our stock culture was lethal in a dose of one-quarter of a culture (the 24 hour growth on an agar slant). More than four times this quantity would prove too great an amount of bacterial protein. Ten lethal doses of a Shiga culture could be easily employed, as the total amount equalled only one-third of the growth on a standard agar slant. However, in all cases control rabbits were used to test the corresponding doses.

Experiment F.—Polyvalent antidysenteric serum of high titer was absorbed twice with Shiga and Flexner cultures by the method described by Gibson. Rabbit 14 was injected subcutaneously with 1, 2, and 3 billion killed bacteria at 5 day intervals. 1 cc. of the modified serum was injected with each dose. Considerable local reaction resulted from each injection. The rabbit showed agglutinins only after the second injection. The titer was low, 1: 50 for both types of bacilli. 1 month after the last injection this rabbit survived four lethal doses of a Flexner culture, injected intravenously. Rabbit 15 was injected in the same way as Rabbit 14, except that the modified serum was mixed with the killed bacteria previous to injection. There was no local reaction. The agglutinin titer was practically the same as in Rabbit 14. 1 month after the last injection this animal succumbed to ten lethal doses of a Shiga culture.

In this series the results with regard to the protection afforded correspond exactly with those in which the whole or unmodified immune serum was employed. Gibson advises the simultaneous employment of vaccine and modified serum, each injected separately. This has not been, in our limited experience, as efficient as the combined whole serum and vaccine as practised by Lucas and Amoss.[8]

The outcome of this series of studies is the conclusion that immune serum added to the killed bacilli, or vaccine, is capable of neutralizing the toxicity of the bacilli, of stimulating the production of agglutinins, and yet of affording only partial protection. All vaccines of this kind may lead, however, to sensitization to horse serum, which is objectionable with respect to large bodies of men.

Oily Suspensions of Dysentery Bacilli as Vaccines.

The use of oils as vehicles for drugs for subcutaneous or intramuscular injection has long been practised. The principle involved is the slow absorption of the drug, unchanged in any way in the preparation, thus allowing the body to accustom itself gradually to the effects of the drug. The method has recently been applied to bacterial vaccines. Le Moignic and Pinoy[20] and Tribondeau[21] suspended typhoid bacilli and subsequently typhoid and paratyphoid A and B bacilli, in certain vegetable oils, the exact nature of which has not been revealed.

[20] Le Moignic and Pinoy, *Compt. rend. Soc. biol.*, 1916, lxxix, 201, 352.
[21] Tribondeau, L., *Compt. rend. Soc. biol.*, 1917, lxxx, 782.

When our studies had reached this point, the *lipo-vaccin* of Le Moignic and Pinoy was brought to our attention by Dr. Carrel. Our first tests with the oily vehicle were made during the summer of 1917. We filtered the French oily vaccine through a Berkefeld candle and thus obtained the oil free from the bacilli. Dysentery bacilli were then suspended in the oil in a proportion such that 1 cc. contained 4 billion organisms.

Experiment G.—Rabbits 16 and 17 were injected subcutaneously, under the shaven skin of the abdomen, with the vaccine in three doses of 0.25, 0.5, and 0.75 cc., corresponding to 1, 2, and 3 billion bacteria. These injections were made at 5 day intervals. No local or systemic reaction was noted. The agglutinin production was later in appearance but was more persistent than with the vaccines of the former experiments. 5 days after the first injection only a trace of agglutinins was demonstrable; 5 days after the second injection the titer was 1:100 for Flexner and Shiga types; 7 days after the third injection the titer was the same, but 1 month later it rose to 1:200 for each type. At this time Rabbit 16 resisted ten fatal doses of a Shiga culture injected intravenously, and Rabbit 17 four fatal doses of a Flexner culture. The controls died in 1 to 2 days.

From these tests it was concluded that the oily suspension answers the requirements of a serviceable vaccine: no local or systemic toxicity is caused by it; agglutinins are formed regularly in good quantities and persist; protection is secured and is still present 1 month after the vaccination.

The exact nature of the oils in the French vaccine being unknown, an attempt was made to substitute a preparation as similar as possible to that in the oily vaccine. Considerable experimentation was necessary to find a substitute. Olive oil was tested, saturated with lanolin to reduce still more the rate of absorption.

Experiment H.—Rabbits 18, 19, and 20 were injected, in a manner similar to that described in the foregoing experiment, with the olive oil vaccine. Extensive swellings developed in all three animals at the site of injection. These subsequently gave rise to large cystic masses containing on section cheesy matter (soaps). Nevertheless there was no systemic toxicity—all three rabbits withstood the injections. The agglutinin production was considerable: 1 month after the last injection the titer was 1:400 for the Flexner and 1:200 for the Shiga type. Rabbit 18 survived ten fatal doses of a Shiga culture at this time.

The olive oil was implicated in the production of the local irritation, possibly on account of the presence of oleic acid. Archard and

Foix[22] experienced similar difficulties and ascribed the local abscess formation to the heating of the olive oil at too high a temperature during sterilization. With respect to agglutinin production and protection this oily vaccine was promising, but the severity of the local reaction precluded its use. Almond oil saturated with lanolin was selected for the next experiment. The results with it were more satisfactory.

Experiment I.—Rabbits 21, 22, and 23 were injected with the lanolin-almond oil vaccine in a manner similar to that of Experiment G. The first injections were not attended by any local reactions; *i.e.*, when 0.25 cc. was employed. Subsequent injections of 0.5 cc. or more, however, produced extensive indurated areas at the site of injection. The agglutinin production was marked: 1 month after the last injection the titer was 1: 400 for the Flexner, 1: 200 for the Shiga type. At this time Rabbit 22 survived ten fatal doses of a Shiga culture and Rabbit 23 four fatal doses of a Flexner culture, injected intravenously. Four control rabbits similarly injected died within 24 hours.

In spite of the promising results from the immunological standpoint, the outcome was unsatisfactory because of the local induration sometimes produced. The almond oil was therefore tested alone.

Experiment J.—Rabbits 24, 25, and 26 were injected in the usual manner. Neither local nor systemic reactions resulted. The agglutinin production, as a rule, for these rabbits had the following titers: 5 days after the first injection, negative for Flexner and Shiga types; 5 days after the second injection, 1: 50 for Flexner and Shiga types; 5 days after the third injection, 1: 100 for both types; and 1 month after the third, 1: 200. Subsequent experiments proved that protection to ten fatal doses of a Shiga culture and four fatal doses of a Flexner culture, injected intravenously, was present at this time.

In view of the absence of systemic reaction and the low degree of local effect, while agglutinin production is stimulated and protection afforded, we may regard the oily vaccine as a preparation possibly suited for use in man.

Whitmore and his coworkers[23, 24] have recently published their results of the study of oily vaccines of the dysentery bacilli and some

[22] Archard, C., and Foix, C., *Compt. rend. Soc. biol.*, 1916, lxxix, 209.

[23] Whitmore, E. R., Fennel, E. A., and Petersen, W. F., *J. Am. Med. Assn.*, 1918, lxx, 427.

[24] Whitmore, E. R., and Fennel, E. A., *J. Am. Med. Assn.*, 1918, lxx, 902.

other microorganisms. Their experiments cover a considerable number of points, including the immunizing effects on rabbits and man and the methods of preparation of the vaccine on a large scale. They employ dried cultures emulsified in a mixture of oil (olive, etc.) and lanolin by grinding in a ball mill, using glass bottles and steel balls.

Immunization by Means of a Single Injection of Oily Vaccine.

The next step in this study was the determination of dosage.

Experiment K.—Several rabbits were injected subcutaneously with a single dose of 1 cc. of plain almond oil vaccine, containing 4 billion dysentery bacilli. Rabbits 27 and 28 were carefully studied with reference to agglutinin production and the following results of slow absorption observed: 8 days after the injection the titer was 1: 50 for Flexner and Shiga bacilli; 1 month after the injection, having fluctuated within this period from 1: 50 to 1: 200, the titer was 1: 100 for either type. Rabbit 28 survived ten fatal doses of a Shiga culture, and Rabbit 27 four fatal doses of a Flexner culture, injected intravenously. Two control rabbits, injected similarly, died within 2 days.

The deduction from these tests is that a single dose of sufficient quantity of the oily vaccine is capable of yielding agglutinins for and providing protection against the dysentery bacilli which endure practically undiminished for at least a month. The experiment was next performed on a monkey.

Experiment L.—0.5 cc. of the plain almond oil vaccine containing 2 billion dysentery bacilli was injected subcutaneously into a monkey (*Macacus rhesus*) weighing 5,050 gm. After 48 hours a slight induration developed at the site of injection, but there was no active inflammatory reaction—no redness or tenderness. The monkey was lively and had no fever. The slight induration persisted for about a week and was then completely absorbed. 7 days after the injection the blood showed agglutinins for both types of dysentery bacilli: for Shiga cultures 1: 400, for Flexner 1: 200.

The study had now progressed to the point where the vaccine was ready to test on man. Several men, physicians chiefly, volunteered. Of the eight volunteers four served to determine the proper dosage and the other four to confirm the results obtained with the first group and to show the practicability of the vaccination method. The results with each individual follow.

F. G. S. was injected subcutaneously with 1 cc. of the oily vaccine, containing 5 billion bacteria. The systemic reaction was slight, consisting of headache, slight chilliness, but no rise in temperature, and lasted for 1 day. The local reaction, however, was severe. There were redness, tenderness, and induration over an area 6 cm. in diameter. This persisted for 1 week, after which absorption became evident; the entire indurated area disappeared in from 3 to 4 weeks. The agglutinin production was as follows: up to the 8th day, negative; on the 8th day, 1: 50 Flexner, 1: 25 Shiga; on the 15th day, 1: 400 Flexner, 1: 100 Shiga; 1 month later, 1: 50 Flexner, 1: 25 Shiga.

J. W. S. was injected similarly. His reactions were slightly more severe than those of F. G. S. The agglutinin production was as follows: up to the 8th day, negative; on the 8th day, 1: 200 Flexner and Shiga; on the 15th day, 1: 800 Flexner and 1: 400 Shiga; 1 month after the injection, 1: 200 Flexner and Shiga.

Hence, in as far as these two tests are concerned, the local reaction may be regarded as too severe to permit the wide use of the vaccine. However, neither of these men lost a day from his work.

On further study, however, it was found that the oil used in the preparation of the vaccine was incompletely neutralized. Hence the next two men were injected with a neutral oily vaccine, to be described later, and at the same time the dosage was diminished to 0.75 cc. containing 3,750,000,000 dysentery bacilli.

G. E. M. showed only a slight systemic reaction. There was no local reaction, except that 6 days after the injection there were slight tenderness and redness which persisted for 3 days and then disappeared. The vaccine was completely absorbed in 9 days. The agglutinin production was as follows: up to the 14th day, negative; on the 14th day after injection, 1: 50 Flexner and Shiga; 1 month after the injection, 1: 25 Flexner and Shiga.

L. E. M. had no local or systemic reaction. The agglutinin production was: on the 7th day after injection, 1: 200 Flexner and Shiga; on the 14th day, 1: 200 Shiga and 1: 100 Flexner; on the 22nd day, 1: 400 Shiga and 1: 100 Flexner; 6 weeks after the injection, 1: 400 Shiga, 1: 50 Flexner.

On the basis of these tests we concluded that 0.5 cc., containing a total of $2\frac{1}{2}$ billion bacilli (equal parts of Shiga and Flexner strains), would be a practicable dose. The following four men were given this amount.

B. G. had no systemic reaction. Locally there was an indurated area, 2 cm. in diameter, which persisted for 2 weeks. It was tolerable and was accompanied by no troublesome symptom. This man is subject to boils, a fact which

may explain a slight infection which developed in the area. The complication was neither painful nor troublesome. The agglutinin production was: on the 7th day after injection, 1: 100 Flexner, 1: 25 Shiga; on the 14th day, 1: 50 Flexner and Shiga; on the 22nd day, no agglutinins for Flexner or Shiga bacilli.

F. H. M. showed a slight systemic reaction. The local reaction was shown by slight redness and moderate induration, which was absorbed in 14 days and was tolerable. The agglutinin production was as follows: on the 7th day after the injection, 1: 50 Flexner and Shiga; on the 14th day, 1: 50 Flexner, 1: 200 Shiga; 1 month after the injection, 1: 50 Flexner and 1: 400 Shiga.

D. R. B. had no systemic reaction. There was an indurated area 4 cm. in diameter, but it was not in any way troublesome and was completely absorbed in 3 weeks. The agglutinin production was as follows: on the 7th day after injection, 1: 50 Flexner, 1: 25 Shiga; on the 14th day, 1: 100 Flexner, 1: 50 Shiga; 1 month after injection, 1: 100 for Flexner and Shiga bacilli.

P. K. O. showed a slight systemic reaction. Induration developed at the site of injection, and there was tenderness of this area for 2 days. The induration was absorbed after 10 days. The agglutinin production was shown to be negative up to the 8th day; on the 18th day after injection it was 1: 200 for Shiga and Flexner bacilli; 1 month after injection it was 1: 100 for both types; 3 months after injection it was 1: 80 for Flexner and 1: 40 for Shiga bacilli.

From a summary of the experiments with the plain neutralized almond oil vaccine in man, it appears that a single injection results in slight or no systemic reaction and a moderate local reaction. The latter consists in the formation of a subcutaneous indurated area, corresponding to the unchanged oil and bacteria, which gradually recedes, 1 to 3 weeks being required for complete disappearance, during which period no inconvenience is suffered. The agglutinin production is indicative of the slow absorption of the antigen. Agglutinins tend to appear after 7 days, to increase as a rule from this time to the 3rd week, and then to persist for a month at least. In one instance an appreciable amount of agglutinin was still present 3 months after the single injection of the oily vaccine.

Mode of Action.

The advantages of the oily suspensions of dysentery bacilli for purposes of active immunization or vaccination depend on several properties. In the final analysis the desired result is achieved by the disintegration and absorption of the contained bacilli. In this respect there is no distinction between the saline and oily suspensions. The chief differences are in the rate of absorption.

In the case of the saline suspensions, disintegration rapidly ensues. The tissues at the point of inoculation are brought under the influence of the concentrated toxic products, and a sharp local reaction follows. The rapid absorption of these products sets up also a marked general reaction.

On the other hand, the suspended bacilli are only slowly yielded up by the oil; hence disintegration is gradual and the local toxic action on the tissues minimized. Moreover, and for the same reason, absorption proceeds slowly, so that the general reaction is either eliminated altogether or greatly diminished; and yet the immunity response is at least as great in the latter instance as in the former, and it may even be greater, since it occurs with less cost to the organism of the host as a whole.

The rate of absorption of the two kinds of suspensions was studied experimentally.

Experiment M.—Rabbit 29. Nov. 14, 1917. Injected subcutaneously with 1 cc. (equals 2 billion dysentery bacilli) of saline suspension vaccine. Nov. 15. Swelling and redness over an area of 5 by 3 cm. The area was aspirated and the drop of fluid obtained examined in a film preparation stained by Gram's method. No dysentery bacilli were seen. There were present a few polymorphonuclear cells, most of them fragmented, and a number of large endothelial cells. Nov. 16. The appearances in the film preparations of the exudate were identical with those of the day before. Nov. 17. No exudate obtainable.

Rabbit 30. Nov. 14, 1917. Injected subcutaneously with 1 cc. of almond oil vaccine (equals 2 billion dysentery bacilli). Nov. 15. 0.25 cc. aspirated from the indurated area caused by the injection. The aspirated fluid was grossly indistinguishable from the oily vaccine. Microscopically, there were numerous Gram-negative bacilli, a few large flat endothelial cells, and a few polymorphonuclears containing numerous bacilli (phagocytosis). Nov. 16. The aspirated fluid showed no change. Nov. 17. The aspirated fluid showed only a trace of oil and a few Gram-negative bacilli and fragmented polymorphonuclears. The bacilli showed lytic changes. Nov. 18. No exudate obtainable.

From this experiment one may conclude that with the saline suspension the bacteria disappear from the site of injection in 24 hours, with the oily vaccine not until after the 3rd day. In the mode of operation of the latter, therefore, slow absorption is the essential factor.

The Shiga bacillus is capable of developing in culture media, besides an endotoxin, a soluble toxin of great potency.[22,25,26] The efficacy of the oil in mitigating the effect of this toxin is shown by the following experiment.

Experiment N.—By growing a toxic strain of the Shiga bacillus in a medium of neutral sugar-free broth to which a protein, such as egg albumin, is added, a toxin is obtained the minimum lethal dose of which varies between 0.05 and 0.1 cc. The sample, Toxin 4, used in the following test was prepared Dec. 1, 1917, and when injected intravenously into a rabbit in a dose of 0.1 cc. caused paralysis of the posterior extremities after 3 days and death on the 4th day. Autopsy revealed the typical intestinal lesions of Shiga bacillus inoculation of the rabbit. The toxin resists drying.

Rabbit 31. Jan. 2, 1918. Injected with ten minimum lethal doses of dried (*in vacuo*) Toxin 4 emulsified in 1 cc. of saline solution and kept at 37°C. for 30 minutes. Jan. 3. Paralysis of posterior extremities; diarrhea. Jan. 4. Found dead.

Rabbit 32. Jan. 2, 1918. Injected intravenously with ten minimum lethal doses of the dried Toxin 4 emulsified in 1 cc. of plain almond oil and incubated at 37°C. for 30 minutes. The animal survived, with no loss of weight or other symptoms. The experiment was repeated with twenty minimum lethal doses of dried Toxin 4 with similar results: the dried toxin in saline solution caused the death of Rabbit 33 in 2 days; the dried toxin in the oil gave rise to no symptoms in Rabbit 34.

Owing to the presence of the oil, the toxin is probably absorbed in subliminal amounts.

Development of the Shiga Antitoxin.

The next experiment bears on the efficacy of the oily vaccine in leading to antitoxin production.

Horses receiving injections of bacteria show the presence of antitoxin only after a considerable period of time and in small quantity. For example, a horse which had been injected with Flexner and Shiga bacilli continuously for $3\frac{1}{2}$ years yielded a serum of which 0.001 cc. neutralized a minimum lethal dose of Shiga toxin, or, on the basis of 100 minimum lethal doses, 1 cc. contains ten units. The limit of neu-

[25] Doerr, R., Das Dysenterietoxin, Jena, 1907, 30 and ff. Kraus, R., and Doerr, R., *Wien. klin. Woch.*, 1905, xviii, 158.

[26] Todd, C., *J. Hyg.*, 1904, iv, 480.

tralization of normal horse serum is 0.5 cc. for four minimum lethal doses of Shiga toxin.

Experiment O.—Macacus rhesus. Nov. 7, 1917. Bled for trial titration. 2 cc. of the serum were mixed with ten minimum lethal doses of Toxin 4, incubated for 30 minutes at 37°C., and injected into Rabbit 35. The rabbit developed complete paralysis and died in about 20 hours. No antitoxin, therefore, could be demonstrated in 2 cc. of the monkey's blood. The monkey was then injected subcutaneously with 0.5 cc. (equals 2 billion dysentery bacilli) of the oily dysentery vaccine and 7 days later similarly with 1 cc. 1 month later the monkey was bled again and 0.1 cc. of serum plus ten minimum lethal doses of Toxin 4, after incubation at 37°C. for 30 minutes, were injected intravenously into Rabbit 36. The rabbit became paralyzed after 48 hours and died on the 6th day (faint trace of neutralization). 0.5 cc. of serum plus ten minimum lethal doses of Toxin 4, injected into Rabbit 37, gave rise to paralysis after 4 days and death on the 6th day (trace of neutralization). 1 cc. of serum plus ten minimum lethal doses of Toxin 4, injected into Rabbit 38, caused no symptoms (complete neutralization). Rabbit 39 (control), injected intravenously with ten minimum lethal doses of Toxin 4, died within 24 hours.

The preceding experiment confirms the fact that in the *rhesus* monkey, an animal closely related to man, antitoxin may be produced as a consequence of injecting the oily vaccine. A similar test was made with human blood.

Experiment P.—J. W. S. had been injected, 2 months before the following test was made, with a single dose of the oily vaccine.

Rabbit 40 was injected intravenously with ten minimum lethal doses of Toxin 4 plus 1 cc. of J. W. S.'s blood serum. The toxin and the serum were kept at 37°C. for 30 minutes before the injection. The animal survived. Of two control rabbits, one, injected with ten minimum lethal doses of Toxin 4, died in 48 hours; the other, injected with 1 cc. of normal human blood serum mixed with ten minimum lethal doses of the same toxin, died in 3 days, after manifesting the typical intestinal symptoms.

The significance of these experiments is clear. The Shiga bacillus, which is the most difficult to employ for vaccination in man because of its toxic effects, is unimpaired in its antigenic properties by the oil. Hence, although no marked reaction is set up, the immunity response is definite. Not only are antibacterial antibodies produced, but in the case of the Shiga toxin, antitoxin as well; and the latter is demonstrable 2 months, at least, after the injection.

Preparation and Administration of the Oily Vaccine.

Commercial vegetable oils, including almond oil, may contain impurities and objectionable quantities of free fatty acids. Hence, to avoid the former one should use only a rectified oil, and, in the case of almond oil, only that of sweet, not of bitter almonds. To eliminate the free fatty acids each specimen of oil should be neutralized. This may be done as follows:

The oil is dissolved in an equal volume of ether. Sodium methylate is prepared, in the meantime, by dissolving 20 gm. of metallic sodium in 100 cc. of dry (absolute) methyl alcohol. The fresh sodium methylate (it will not keep for more than a week) is added to the dissolved oil to the point of neutralization, a few drops of an alcoholic solution of phenolphthalein being used as indicator. If by mistake an excess of the alkali is added, the harm may be undone by adding more of the original oil. The next day the neutralized oil is filtered by suction or centrifuged to remove the precipitate of soaps, and the ether is evaporated by warming. It is kept in the refrigerator for 1 week, animal charcoal is added, and it is again filtered. It is then sterilized by autoclaving for 45 minutes at 15 pounds pressure.

The oily vaccine is prepared in the following manner. Plain agar cultures in Blake bottles are made of the Shiga, Flexner, and Y bacilli. 24 hour growths are washed off in normal saline solution, usually 5 cc. of saline solution to each bottle being sufficient. The suspension of each strain is thoroughly shaken and a portion set aside for counting, which is done by Wright's method. In the meantime the saline suspensions are transferred to wide centrifuge tubes, 2.5 by 10 cm., and are centrifuged for 20 minutes at about 1,800 revolutions per minute. Since the cotton stopper may be jammed to the bottom the cotton is replaced by sterile tin-foil, folded over the edge of the tube. At first, drying *in vacuo* was attempted, but it proved inconvenient, and centrifugation was substituted to obtain the bacterial mass, with good results. Almost all the saline solution can be pipetted or poured off, leaving a densely packed sediment of bacteria. The count of the bacteria and the amount of saline solution having been determined, it is a simple matter to add sufficient oil, replacing the saline solution, so that 1 cc. will contain $2\frac{1}{2}$ billion

Shiga and $1\frac{1}{4}$ billion each of Flexner and Y bacilli. The total content in 0.5 cc. of the vaccine should be $2\frac{1}{2}$ billion dysentery bacilli in aliquot parts of the Shiga and Flexner groups of organisms. The required amount of oil is added to the centrifuged bacterial mass, and by means of a bent glass rod an oily suspension is made. The suspension is then transferred to receptacles containing glass beads. Glass beads must always be included, irrespective of the quantity of vaccine in a container. They are the essential factor upon which a proper suspension depends. No preservative is added, as it has been determined that it is without action in oils. The oily vaccine is heated to 60°C. for $\frac{1}{2}$ hour. Tests are made for sterility and for its effect in the rabbit. The vaccine is stored in the refrigerator.

The oily vaccine is warmed slightly before use, in order to make it more fluid, and shaken thoroughly. The skin over the deltoid region is cleansed with alcohol, tincture of iodine is painted on it, and 0.5 cc. of the vaccine is injected subcutaneously. Care is taken to avoid intracutaneous or intravenous injection.

DISCUSSION AND SUMMARY.

The purpose of the present investigation was to determine a practical method of vaccination against bacillary dysentery.

It has been emphasized that the toxicity of the dysentery group of organisms, especially that of the Shiga bacillus, is such as not to permit of their employment in simple saline or aqueous suspensions. If, on the other hand, their toxicity is removed by the addition of certain chemicals, the antigen of the bacilli is so changed as not to be suitable for immunization purposes.

The toxicity of the bacilli can also be diminished by the addition of immune serum. There is no essential difference in the result whether unmodified serum is used or that modified by Gibson's method. The use of serum with vaccines cannot be recommended, in spite of the relative non-toxicity of such mixtures. The specific immunity response is reduced, while the parenteral injection of horse serum in large groups of men is objectionable because of the serum sensitization which it produces.

It has been shown that a certain vegetable oil, almond oil, (and this statement may apply to a number of non-irritating, absorbable oils) is capable of overcoming many of these disadvantages.

The oil acts as a passive agent in merely suspending the bacteria without altering their properties. The slow absorption of the suspended bacteria from this vehicle mitigates the toxic effects of the dysentery bacilli. At the same time it does not interfere with the immunity response—antibacterial and antitoxic.

If the absorption is too slow, however, as in the instances in which lanolin was added, less satisfactory results follow. The proper rate of absorption is as important a factor as the proper vegetable oil. The latter condition has been emphasized in the consideration of the effects of an objectionable olive oil.

The neutralization of the oil should be complete. F. G. S. and J. W. S. suffered from severe local reactions, the results of the local deposition of soaps, which are difficult of absorption.

As a result of the slow absorption of the dysentery bacilli from the oily suspension only slight local and general reactions follow, and it is possible to give at one time and in a single dose a sufficient number of the killed dysentery bacilli to incite a high degree of immunity.

The precise series of events following the injection of the oily vaccine are: During the 1st day, an erythematous area develops at the site of injection, which is not especially painful. There may be a slight systemic reaction, consisting in headache and slight chilliness. After 24, sometimes after 48 hours, an induration appears at the site of injection, varying from 2 to 4 cm. in diameter, which remains unchanged for a few days, then begins to recede, complete absorption requiring from 1 to 3 weeks. The induration remains localized and has no tendency to break down. It causes no inconvenience.

During the period of absorption the organism of the host continually receives antigen. The rule of immunology is that while antigen is circulating the antibodies are usually not demonstrable; it is only after all the antigen disappears that the antibodies appear in greatest concentration. Since the vaccine is slowly absorbed, the reasons for the delayed appearance of antibodies, as well as their persistence, become obvious.

CONCLUSIONS.

The preceding study seems to emphasize the advantages of a bland oily medium for suspending dysentery bacilli for purposes of active immunization or vaccination. The experiments on animals and a small number of tests on man indicate that the single injection of an almond oil suspension of the Shiga and Flexner groups of dysentery bacilli suffices to afford protection as indicated by the appearance in the blood of definite specific antibodies for each group of the bacilli, and by the protection of animals from otherwise lethal doses of the living organisms or their toxic products. The extent to which vaccination should be applied to man will depend on circumstances and conditions still to be defined, but the method appears to be wholly practicable. The introduction by Le Moignic and Pinoy of the oily medium for suspending killed bacteria for immunization purposes marks a definite advance in the technique of bacterial vaccination.

COCCIDIOSIS IN YOUNG CALVES.

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Plates 1 to 3.

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Among protozoan parasites coccidia have received considerable attention because the asexual and sexual cycles are readily available for study. They have served to demonstrate the remarkably complex life cycle of certain groups of protozoa. They also possess a certain practical importance since they may produce diseases, at times of epizootic proportions, among domestic animals. Coccidiosis as a disease in cattle was first brought forward in 1892 by Zschokke[1] and Hess[2] who observed it in Switzerland. The name *rothe Ruhr* was suggested by the discharges of blood in the feces. Before this Zürn[3] in 1878 had reported coccidia in the intestines and mesenteric glands of a calf which died as a result of a severe enteritis. Since 1892 coccidia have been found in cattle by various observers, especially in tropical and subtropical countries and to a slight extent in temperate climates (Denmark, Russia, France, Italy, and Germany). So far as it is possible to consult the original literature, no studies of the cases found were made beyond the microscopic examination of the feces except by Züblin[4] who undertook a fresh investigation of certain cases of *rothe Ruhr* in Switzerland in 1906 and whose work is referred to farther on.

In the United States up to 1911 the presence of coccidiosis had not been noted. In a general article on the subject Crawley[5] writes as follows:

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[1] Zschokke, E., Beobachtungen über die rothe Ruhr, *Schweiz. Arch. Tierheilk.*, 1892, xxxiv, 1, 49.

[2] Hess, E., Die rothe Ruhr des Rindes. (Dysenteria hæmorrhagica coccidiosa.), *Schweiz. Arch. Tierheilk.*, 1892, xxxiv, 105.

[3] Zürn, F. A., Vorträge für Thierärzte, 1878, i, No. 2.

[4] Züblin, E., Beitrag zur Kenntnis der roten Ruhr des Rindes (Dysenteria coccidiosa bovis), Inaugural dissertation, Zurich, 1908.

[5] Crawley, H., *Twenty-Seventh Ann. Rep. of Bureau of Animal Industry, U. S. Dept. Agric.*, 1910, 488.

"*Coccidium zürni.*—This coccidian is considered to be the cause of red diarrhea of cattle. The disease has been noted in Europe, where outbreaks of a similar character have also occurred among sheep. The lethality, however, for cattle seems to be rather low, varying from 2 to 4 per cent. As it is the intestinal cells which are attacked by the parasite, the mucosa of the intestine becomes stripped off in places, resulting in extensive hemorrhage into the lumen, which causes the red diarrhea. In fatal cases, death results within 2 days. Most of the recorded cases have occurred in Switzerland, in summer and autumn. Dampness favors the disease, probably for the reason that in dry weather the encysted infective stages of the parasite become desiccated, and thus many are killed."

In September, 1915, Schultz[6] stated that coccidiosis existed among cattle in the Northwest of the United States without, however, supplying any detailed data.

Dr. Schultz kindly sent some slides of what appeared to be washed feces to us recently. A careful examination by two observers independently resulted in the finding of a few undoubted oocysts. These would demonstrate the existence of the parasite in the Northwest. In other slides in which schizonts and merozoites were supposed to be present only desquamated epithelium was found. The character and intensity of the western disease needs, therefore, further elucidation.

In the prosecution of certain researches upon the diseases of calves in New Jersey, coccidiosis was encountered unexpectedly in association with other infectious diseases. Though the material available for study was not extensive, it became evident that coccidiosis might by itself prove fatal to calves, and the subject was deemed of sufficient importance, therefore, to be discussed independently of other associated calf diseases.

General Characters of the Disease.

Epidemiology.—The first case containing coccidia was autopsied April 14, 1917. Other cases followed during the spring and summer. A few coccidia were found in a calf as late as December 2. The severest, fatal cases occurred in summer. The invasion probably begins soon after birth and continues over a period of 6 to 8 weeks at the

[6] Schultz, C. H., Observations among dairy cattle, read at the Annual Convention of the Washington State Dairymen's Association, Snohomish, December 15-17, 1915.

longest, unless death occurs in the meantime from this or other affections. Blood in the stools, being the characteristic sign which probably appears when the first crop of oocysts is being discharged, occurred anywhere from 3 to 6 weeks after birth. The fact that the affected calves were not under our immediate care makes any statement bearing on the clinical course subject to corrections. In several severe cases blood in the feces was not reported.

Evidence of active multiplication of coccidia in cattle older than 3 months is not at hand. The disease appears to exert itself chiefly upon the very young, the older animals having acquired a certain resistance.

Infectiousness does not seem to be very great. The calves were kept together in lots of six or less in small indoor enclosures and were allowed to run together in small yards. Assuming that the oocysts passed in the feces become infectious within a few days in warm weather, there was a striking difference in the susceptibility of different calves of the same lot. The presence of pneumonia or interstitial nephritis did not seem to affect the susceptibility in one direction or the other.

Symptoms.—The clinical examination of the calves was not carried out in any systematic way because they were not at any time during life on the Institute grounds. It will be noticed by consulting the protocols that certain animals were affected with pneumonia and with a sclerosis of the kidney which complicated a study of those manifestations due directly and exclusively to the coccidiosis. The discharge of blood in the feces was the only characteristic sign. In nearly all these cases the oocysts were found in the masses of blood (Fig. 1). The few observations made during the life of the calves are noted under each case.

Inasmuch as Züblin had under observation cases at first uncomplicated with other infections, his clinical notes are of value to us. He does not mention young calves as subject to this disease. His cases were chiefly pastured animals and in one herd of ten head of cattle, all of which were affected, the ages ranged from $\frac{1}{2}$ to 2 years.

According to Züblin, the first sign of disease is the passage of blood clots, about 10 to 15 cc., in normal feces by clinically normal animals. 1 or 2 days later blood is more abundant, defecation more frequent. The case may now return to normal

or it may grow worse. Usually the older animals recover promptly. In younger animals the quantity of blood passed may increase up to a man's fist in size. The tenesmus becomes more intense and the feces tend to become thin, foul smelling, the temperature rises, respirations are increased in number, and the appetite is abnormal. From the 5th to the 8th day the admixture of blood in the feces is replaced by mucus and masses suggesting croupous inflammation of the large intestine. The feces are watery and putrefactive. All other symptoms are accentuated and fever is present. With the loss of blood and refusal of food the animal becomes very weak and death occurs often as early as the 7th day. The mortality in Züblin's cases was 5 per cent.

The symptoms in his cases point to septic infection as the probable cause of death. It is obvious that the mortality rate in this disease will depend largely upon the presence of septic organisms in the herd and their relative degree of virulence, for the coccidia themselves do not cause inflammatory reactions but simply open the way for bacterial infection.

Diagnosis.—The feces were examined directly when blood or mucus or both were accessible, by placing a particle of the suspected material on a slide with or without a drop of salt solution and examining under a cover-glass with low and high powers. The oocysts are sufficiently characteristic to be identified under high dry objectives, and they are often recognizable under a low power. Drying and staining should not be resorted to.

When the feces are free from blood and coccidia are not detected directly, the following method for concentrating the feces which is a simplified modification of Hall's method[7] and which was used in our examinations will be found useful:

A mass of feces is thoroughly shaken in a closed Mason jar partly filled with water, until well broken up. Then the jar is completely filled with water and the feces are permitted to settle. The water is poured off and the jar again filled with water. The sedimentation, decantation, and washing are continued until the water is nearly clear. In this way the particles lighter than water are removed. The sedimented feces are next mixed in a jar with about enough water to fill a cylinder in which they are to be sedimented and are poured through a tea-strainer held over the cylinder. By stirring the portion of the feces held back in the strainer and by dipping the bottom of the strainer up and down in the water after it nearly reaches the top of the cylinder, oocysts that have been held back are permitted to pass through. After the feces have settled to the bottom, a sample

[7] Hall, M. C., A comparative study of methods of examining feces for evidences of parasitism, *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 135*, 1912.

is taken with a pipette of wide caliber, mounted, and examined. By this method a few oocysts were brought to light in normal stools from calves apparently in good health.

After death the gross appearance of the large intestine does not offer any characteristic features for the recognition of the invasion. Two indications might be looked for: a change in the appearance of the mucosa due to the mechanical presence of large numbers of coccidia, and changes due to pathological alterations of the invaded tissue. As regards the first clue nothing is deducible from our observations. It may be stated here that in other species indications may be present. In a case of coccidiosis of the small intestine of a sheep seen by one of us many years ago, the foci were readily detected by the unaided eye. They were circular, opaque, whitish, slightly elevated patches up to a centimeter in diameter contrasting sharply with the normal pinkish mucosa. The slight thickening of the patch was due to the fact that every epithelial cell of the villi contained several coccidia, and as a result the affected villi became giants as compared with the normal. It should be stated that this animal had been killed and postmortem changes were absent. With regard to the second indication, the presence of one or more gross pathological changes, it may be said that the severest of the cases observed died in hot weather and came to autopsy when postmortem changes were well under way. Congestions and scattering punctiform hemorrhages into the mucosa were the most definite alterations noticed. The diagnosis will in all cases depend on the microscopic examination of the feces, or on sections of the large intestine, or on both combined.

The Parasite.

The Oocysts in the Feces.—In the course of the work it became evident that there were two species of coccidia infesting the calves. The species first encountered and present in large numbers is distinguished by a small oocyst usually elliptical but occasionally ovoid or circular in outline. The wall is double contoured, uniform in thickness, and as a rule thinner than the wall of the oocyst of the other species. Oocysts were encountered in which the protoplasm completely filled the cyst wall. All stages to that in which the protoplasm

was contracted to a circular mass removed from the cyst wall or touching it only at one side have been encountered. When the protoplasmic mass is well contracted it is located as a rule near the center of the cyst, yet it may be in contact with one side of the wall or nearer one end than the other. The cytoplasm contains highly refractive granules varying in size which may be uniformly or irregularly distributed through it. The nucleus is sometimes visible. Fifteen oocysts were measured from Calf E. The length ranged from 14.6 to 20.5 microns, and the width from 12.3 to 16.4 microns. Eleven oocysts were measured from Calf 102. The length ranged from 13.1 to 28.7 microns, the width from 12.3 to 20.5 microns. Ten cysts were measured from Calf 121. The length ranged from 16.4 to 20.5 microns, the width from 13.1 to 16.4 microns. The average length of the above thirty-six oocysts was 18.6 microns, the average width 14.8 microns.

The second species characterized by large ovoid oocysts was first encountered in Calf 92, dying June 6, and thereafter in most cases, either in the feces, or in fresh scrapings of the mucosa, or in sections. The oocyst is distinctly ovoid in shape. Sometimes the cyst wall is brownish in color, at other times it is colorless. The wall is thickest at the broad pole and very gradually diminishes in thickness to the small pole. The difference at the two poles is not great yet distinctly noticeable. Some cysts occur, however, in which the wall is uniform in thickness. There is no evidence of a micropyle.

The cytoplasm as a rule is contracted into a circular mass located at the middle or in the broader portion of the cyst (Fig. 6). It occupies two-thirds to three-fourths of the space within the wall and comes almost in contact with the sides. The cytoplasm is filled with large highly refractive granules. The nucleus is not visible. Thirteen oocysts were measured from Calf B. The length ranged from 25.8 to 41.8 microns and the greatest width from 17.2 to 24.6 microns. Fifteen cysts were measured from Calf E. The length ranged from 28.7 to 32.8 microns, the width from 16.4 to 20.5 microns. The average length of the above twenty-eight oocysts was 29.9 microns and the average width 19.9 microns.

Development of the Oocysts in Cultures.—In order to determine certain points in the development of the oocysts, feces, blood, and mucus

containing them were streaked over the surface of agar plates.[8] The surface of the plates was kept moist by adding from time to time a little distilled water. They were kept in a moist chamber at room temperature.

In the large, ovoid species the mother sporoblast or oocyst produces four spores without leaving behind a so called *Restkörper*. The spores are elongated, nearly elliptical in outline, circular in cross-section, and taper a little toward one end. The spore is surrounded by a thin, single contoured membrane which is thickened to form a cap-shaped body at the smaller pole. There is a large *Restkörper* within the spore. It is elliptical or fusiform in shape and lies against the wall of the spore, sometimes in a space formed between the sporozoites, in which case it occupies a diagonal position with respect to the spore. The *Restkörper* contains rather large, round, highly refractive granules of approximately the same size.

There are two sporozoites in each spore and these with the *Restkörper* completely occupy the space within the limiting membrane. They are broad and rounded at one end and taper toward the other end. The broad ends of the sporozoites are at opposite ends of the spore and the contact between the two is marked by a line running diagonally across the spore. The position of the nucleus varies somewhat. It is either located nearer or in the broad end of the sporozoite. It appears as a small halo, enclosing a minute, round nucleolus. The cytoplasm is minutely granular and is uniform in appearance throughout the sporozoite. There is little variation in the size of the spores. Eight spores were measured in the cysts. The length ranged from 16.5 to 17.1 microns, and the width from 7.2 to 7.6 microns.

In the small species with ellipsoidal oocyst four spherical sporoblasts form which become elliptical in outline and develop into the spores (Figs. 2 and 3). These are elongated, elliptical, and, as in the large species, they taper a little at one end. The spore is circular in cross-section. It is surrounded by a thin, single contoured membrane which is thickened into a minute cap at the small pole. There is no *Restkörper* left behind in the formation of the sporoblasts. There is no

[8] This medium consisted of 20 gm. of agar-agar, 5 gm. of sodium chloride, and 1,000 cc. of distilled water. The agar-agar is cut up, tied in a gauze bag, and washed for 2 hours in running water before the medium is made up.

Restkörper in the spore. The sporozoites are the same shape as in the large species. They are round at the broad end and taper toward the other. In one instance the tapering end was seen to be pointed. In the middle of the sporozoites or possibly more frequently in the large end is generally found a rather large, more or less well defined mass of very minute granules. Elsewhere the protoplasm is homogeneous in appearance. The nucleus is located nearer the broad end and consists of a small, round granule surrounded by a halo. The line separating the sporozoite runs diagonally across the spore. The sporozoites completely fill the spore.

The spores do not vary much in size. They are much smaller than those of the large species. Seven spores were measured. The length ranged from 9.9 to 11 microns, and the width from 5.3 to 5.7 microns. The results of the various measurements are brought together in Table I.

TABLE I.

Species.	Oocyst.		Spore.	
	Average length.	Average width.	Length.	Width.
Ovoid.	29.9 microns.	19.9 microns.	16.5–17.1 microns.	7.2–7.6 microns.
Ellipsoidal.	18.6 "	14.8 "	9.9–11 "	5.3–5.7 "

Studies to determine the exact time required at certain favorable temperatures from the discharge of the oöcyst to the maturation of the sporozoites have not yet been made. Approximate estimates based on cultures and subject to revision indicate that in midsummer this portion of the cycle of the ovoid type covers about 5 days. Similar estimates indicate a somewhat longer period for the ellipsoidal type.

Von Wasielewski[9] states that the oocysts of *Eimeria cuniculi* vary in size and form within wide limits. Cysts occurred the volume of which was only a fraction of the average size. The ripening of these small cysts was normal. Similar variations in size were encountered by him among the oocysts of *Diplospora bigemina* from the cat's intestine. Züblin likewise refers to variations in size corresponding fairly well with our types. Thus in rare cases, he states, the width was 20 microns,

[9] von Wasielewski, T., Studien und Microphotogramme zur Kenntnis der pathogenen Protozoen, Leipsic, 1904, 1. Heft.

the length 30 to 35 microns. The largest number were roundish with a mean diameter of 12 to 15 microns. Nothing is stated of morphological differences between these extremes. It may therefore be claimed that the inference that there were two species of coccidia in the calves is not called for. Pending further investigations it may be pointed out that forms intermediate in both shape and size between the two types of oocyst were not encountered. The small ellipsoidal form was the numerically predominating type and in a few cases the one exclusively seen. The presence of a *Restkörper* in the large, and its absence in the small oocyst is noteworthy. Seeing these two forms in the same field of the microscope, the observer is led to the conclusion that the burden of proof that they are variants of the same species must rest with the one who supports such a view. Anticipating somewhat, we may state that careful study of the sections did not enable us to distinguish between these species in the schizogonic cycle. This may have been due to the relative scarcity of the larger parasite in the material at hand. In any case the final decision will require a study of fresh material on a relatively larger scale.

Among those who have reported on the presence of coccidia in cattle, Guillebeau,[10] Züblin, and Jowett[11] have attempted cultures of the oocysts. Züblin describes the development of the four spores and eight sporozoites as we have found it in the ellipsoidal form. But in addition he describes forms as going through the stages of schizogony in his cultures—forms which may have been other protozoa, such as amebæ, or fungi and of which we also have found multiplying forms in cultures. Further material will be needed to determine whether Züblin actually found a hitherto undescribed stage in fecal cultures or whether he simply described stages of coexisting organisms. Jowett[11] (South Africa) describes a case of bloody dysentery in a calf 3 months of age. The coccidia were round or subspherical. Distinctly oval cysts were rare. The smallest measured 14.4 by 12.8 microns, the largest, 27.2 by 20.8 microns. No details are given except that four sporocysts form, within which sporozoites appear.

The Parasite in the Mucous Membrane.—Owing to the fact that the material on hand coming from animals killed was meager and that the heaviest invasion occurred in animals which died and in which postmortem changes were under way, any detailed description of the stages of the cycle within the host will be postponed. The barest outline must suffice here.

It may be said, however, in spite of the deficiency of material that nothing has been seen which suggests that the coccidia encountered depart in any significant way from the cycle as mapped out for such a well known coccidium as that of the rabbit.

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[10] Guillebeau, cited by Züblin.[4]

[11] Jowett, W., Coccidiosis of the fowl and calf, *J. Comp. Path. and Therap.*, 1911, xxiv, 207.

It is believed that the stages that have been encountered and described belong to the small elliptical species, for the following reasons: first, the small species, as shown by the number of oocysts in the sections, occurs in very large numbers whereas the large species is relatively rare; and second, no anomalies have been encountered leading one to believe that stages of two forms so different in size were being studied.

The earliest stage observed in epithelial cells is roundish or elliptical in outline. The nucleus is centrally or somewhat eccentrically situated and consists of a well staining karyosome surrounded by a halo usually ill defined. However, a halo is not present with all nuclei. The cytoplasm stains lightly and is alveolar in structure. The roundish forms measure about 6 microns and the elliptical ones 5 to 6 microns long and 4 to 5 microns broad.

Larger uninuclear forms were encountered which the evidence favored interpreting as schizonts. They are elliptical in shape or often compressed laterally in the epithelial cell, then presenting an oblong appearance with the sides more or less straight. The cytoplasm shows the same, or approximately the same structure as that in the multinuclear schizonts and there are no metachromatic granules present. These occurred in crypts where schizogony was general and where there was no definite evidence of sporogony. They measure 8 to 10 microns long and 6 to 7 microns broad.

Forms containing two nuclei are circular or elliptical in outline, there is no cell membrane visible, the cytoplasm is alveolar, and the nuclei consist of a karyosome which may or may not be surrounded by a halo. The elliptical forms measure 5 to 7 microns long by 4 to 6 microns broad.

Forms containing from three to seven, nine, and eleven nuclei have been observed but no attempt has been made to determine the number of nuclei in more advanced forms than these. They are circular or elliptical in form and devoid of a cell membrane. Some schizonts stain more deeply than others and the cytoplasm appears more compact, being almost homogeneous. However, as a rule the cytoplasm stains lightly and is alveolar in structure. The daughter nuclei are located at or toward the periphery of the parasite. Typically the nuclei show a dark staining karyosome surrounded by a rather ill defined halo. In some instances a halo about the karyosome cannot be distinguished.

The next stages seen are schizonts in which the cytoplasm has divided to form the merozoites. The merozoites, as shown in Fig. 5, are elongated, banana-shaped bodies usually lying with long axes nearly parallel and enclosed in a vacuole of the host cell. The nucleus is located at the middle. The entire mass representing the schizont measures 9 to 11 microns. The number of merozoites varies; five intact groups contained 13, 18, 18, 18, and 26 merozoites.

The gametes are usually found among schizonts in a later stage of the parasitism. Macrogametes, microgametocytes, and oocysts may be found in the same tubule. The earliest stages of the gametes it has been impossible to distinguish clearly from young uninuclear schizonts. The cytoplasm of forms believed to be

macrogametes, however, is more compact and stains more deeply than that of the schizont. Soon the enlarging body with nucleus remaining single, the circular or plump oval form, and the presence of metachromatic granules give a specific character to the macrogametes. There is no cell membrane. The nucleus as a rule is eccentrically located. It consists of a karyosome surrounded by a halo which in some instances is very large having a diameter of more than half that of the cell. Stages from 4 to 10 microns in diameter have been studied. Forms 6 microns in diameter have been seen containing metachromatic granules, although there seems to be some variation in the time at which these granules appear, larger forms having been seen which showed none or only very few. The granules are round and vary greatly in size not only in different individuals but also in the same one. They have also exceptionally been found persisting in the oocysts after the cytoplasm has retracted from the enclosing wall. In the largest macrogametes a cell membrane is present and the nucleus may be indistinct.

At a fairly early stage the nucleus of the microgametocyte has divided and many coccus-like bodies which stain intensely in basic dyes are found arranged uniformly or irregularly at the periphery of the cytoplasm. These nuclei of the future microgametes gradually change into very fine, hair-like, slightly wavy bodies which project on all sides from the cytoplasm. Well advanced microgametocytes are circular or elliptical in shape. The cytoplasm is more or less vacuolated and stains irregularly. Those of a circular shape measure 7 to 9 microns.

The development of the shell of the oocyst takes place gradually. The oocyst is first surrounded by a very thin membrane. Later a somewhat thicker single contoured membrane is present which stains with acid stains and increases in thickness until the fully developed, highly refractive, double contoured cyst wall is formed. When the enclosed cytoplasm has retracted from the cyst wall it is seen to be still surrounded by a delicate membrane. Various conditions of the oocysts are found in the epithelial cells, in the lumen of tubules, or in the midst of dislodged epithelial cells. The wall is often crumpled. The cytoplasm may completely fill the space within the cyst wall, may be slightly retracted from it, or may be contracted to a spherical mass occupying approximately a central position. The cytoplasm is most often dense and presents a minutely granular appearance, but in some instances it is alveolar in structure. Metachromatic granules are sometimes present in the peripheral portion of the cytoplasm. The nucleus as a rule stains well and uniformly.

The parasite acts on the host cell by absorbing its nutrient fluids and encroaching on the cytoplasm. There is, however, no change in the cytoplasm immediately surrounding the parasite, leading one to believe that the parasite does not exert a digestive or coagulative influence on it. In rare instances the parasite is surrounded entirely or in part by a clear space, but this has come about through a shrinking of the parasite. This condition is rare in material studied from calves that had been killed and the tissues fixed at once, but in material from calves

that died, in which the tissues were subjected to postmortem changes, it is common, and the smaller size of the schizonts and the compact condition of their protoplasm indicate that it has been brought about by shrinkage.

The nucleus of the host cell may not show any changes at first. Later its affinity for stains may be reduced or almost completely destroyed, and the chromatic masses may be reduced in number or disappear entirely. When a parasite lies contiguous to the nucleus, even though it is an early stage occupying only a small fraction of the cell, the nucleus is compressed and may even present a crescentic form.

Pathology.—The seat of the disease in the calves was the large intestine. In the small intestine scattering tubules were found invaded by one or a few parasites, rarely by many, even when the large intestine was severely involved. The forms encountered were oftener gametes than schizonts. In some tubules both sexual and asexual stages were intermingled.

In the large intestine the infection appeared at first in circumscribed foci. These foci tend to grow larger and in some cases coalesce with one another. Usually a given stage, such as schizogony, prevailed in a focus. Later both sexual and asexual forms were found together. In the same tubule may then be found fully developed merozoites, macrogametes, microgametocytes, and oocysts. The fertilized oocysts are probably discharged very promptly, for they are not abundant in the tubules. While schizogony is going on, oocysts may be discharged in large numbers in the feces. This was seen in Calf 121.

Not infrequently there may be seen between tubules thoroughly infected with schizogonic forms, scattering empty shells of oocysts which are embedded in the intertubular tissue. They are not carried there by manipulations, such as section-cutting, since some of them are within phagocytic cells. These shells represent aberrant parasites which have migrated through the epithelium, have gone through the various stages, and cannot be discharged outward. These aberrant cysts are relatively rare but wherever they occur in association with active schizogony in the tubular epithelium they prove that the disease started in an earlier invasion and that the present schizogony is either the result of fresh infection or else it represents a schizogony continuing from the beginning of invasion.

The topography of the invasion and the stages found in a focus give us a tentative explanation of the way in which the infection spreads. A sporozoite entering a tubule of the large intestine produces about sixteen merozoites which are discharged into the tubule and which infect the neighboring cells. These produce each sixteen merozoites which spread to neighboring tubules centrifugally. If the invasion is heavy and a large number of foci started, these may coalesce and form large patches. When the multiplication has gone on for a time the cycles intermingle and various stages, as stated above, will be found in the same focus.

The relative immunity of the small intestine as compared with the large intestine merits attention. In rabbits and birds (sparrows, hens, turkeys, and pheasants) the principal seat of invasion is the small intestine, and only secondarily the large intestine and ceca. Evidently the conditions in the small intestine are unfavorable in calves. Whether the difficulty is mechanical or physiological, that is, due to the active movements of the tube or to the digestive ferments, remains to be determined.

A more interesting problem in immunity is the self-limitation of the disease in a number of cases and the apparently complete immunity of some calves penned with the severe cases. On page 106 are given brief notes of certain calves which had bloody evacuations associated with large masses of oocysts and which recovered completely. Other calves associated with them remained unaffected and samples of feces concentrated before examination by washing and sedimentation did not reveal any coccidia. It is conceivable that schizogony is a self-limited process. If this is true, immunity would play no important part and the extent of the disease would depend upon the size of the infecting dose. Inasmuch as acquired immunity does play a certain part in the diseases due to protozoa living free in the blood and in blood cells, it cannot be at present ignored for coccidiosis. For practical purposes the infecting dose should be kept as small as possible, if it cannot be entirely eliminated, until its relation to the severity of the resulting disease has been more definitely formulated.

The injuries to the host caused by coccidiosis refer to changes in the mucous membrane directly affected and to remoter, more indirect disturbances. The local lesions caused by the invasion of epithelial

cells in the early stages are insignificant. The host cells retain their normal appearances, except as to size, and inflammatory reactions are absent. When the parasites have become widely diffused and occupy most of the cells, a number of changes are evident. Under a low power the tubules are no longer normal in form. Some appear missing. Under a high power the missing tubules are accounted for by the fact that most if not all of the epithelium has disappeared and the lumen is filled with cell elements chiefly polynuclears. Owing to the absence of a surface epithelium in the material available it is not clear that this epithelium has been invaded, but the presence of cell parasites in tubules up to the surface makes it probable that it also is attacked sooner or later. The loss of surface epithelium accounts for the small masses of leukocytes and fibrin and red blood corpuscles which cling to the denuded surface in places and represent an exudation and also the source of the hemorrhages (Fig. 7). These masses have embedded in them many oocysts. The hemorrhage is thus the aggregate of many small hemorrhages from the capillaries near the surface and this explains why the source of the blood is not macroscopically evident at autopsy.

A further lesion found in the advanced cases is a complete necrosis of a limited territory of the mucosa which involves up to ten or more tubules. In this focus cell nuclei have either completely disappeared or are broken up into fragments or pyknotic. The cytoplasm takes a distinctly reddish tint with eosin.

The more remote disturbances due to intestinal coccidiosis may be looked upon as due to infectious agents gaining entrance into the lymph and blood vessels of the denuded and only feebly reacting mucosa. Owing to the frequent association of pneumonia with the cases studied, injuries other than local are not definitely ascertainable. The pneumonia with one exception was associated with a microorganism to be discussed at another time as the probable etiological factor. In the exceptional case the pneumonic lesions were associated with streptococci and staphylococci. Of the five cases in which large numbers of coccidia were found either in the feces or in the epithelium of the large intestine or in both, probably three died as a result of the coccidiosis. Further studies upon calves kept under careful clinical observation will be needed to properly evaluate the disease as in itself a cause of death in this climate.

Brief Notes on Individual Cases.

Calf 34.—Red and white, female. Born Feb. 23, 1917.

Mar. 28. Coughing considerably. Reported to have had diarrhea several days ago. Mar. 30. Temperature 40°C. Apr. 6. Thin and languid. Cough and dyspnea. Feces liquid, of a greenish yellow color. Abdomen distended. Apr. 10. Temperature 40°C. Skin dry; hair roughened. Yellowish mucous discharge from nose. Discharges watery, with mucus tinged with blood. Apr. 12. Temperature 39.8°C. Growing thinner. Coughing readily induced. Fecal discharges are soft and contain clots of blood. Apr. 13. Blood passed in feces.

Apr. 14. Killed and autopsied. Weight 80 to 90 pounds. Besides consolidation of the cephalic half of lungs which will be discussed more fully at another time, there were no noteworthy lesions. The large intestine contained a rather offensive pea soup-like fluid and the flask-shaped glands below the ileocecal valve were distended with whitish mucus-like masses, but no lesions were detected to account for the blood passed in stools.

Histological examination of pieces of tissue from the small and large intestines fixed in Zenker's fluid and alcohol showed the following: At various levels of the small intestine a few coccidia are detected. They occur in groups. One to four may be seen in a tubule among many tubules not invaded. The distal portions of the villi appear to be free. The parasites are mostly in the sporont stage, as macrogametes, microgametocytes, and oocysts. The oocysts measure 20 to 22 microns long and 12 to 14 microns broad. A small number of tubules contain leukocytes both neutrophil and acidophil.

In sections from four different regions of the large intestine one focus was found within which the epithelial cells of the tubules were nearly all invaded by schizonts (Fig. 4). In intertubular tissue several empty oocyst shells were found.

Calf 36.—Red and white, female. Born Feb. 21, 1917.

Attention was first drawn to this case on Apr. 12 when it was noticed that large amounts of blood had been passed per rectum. Temperature 39.3°C. Apr. 13. Blood still present in feces and staining the anal region, tail, and legs. Temperature 39°C. Apr. 15. Calf reported very sick by attendant last evening. Found dead this morning.

The autopsy was not made until early, Apr. 16. The adipose tissue was abundant. The only recognizable lesions were localized congestion associated with punctiform hemorrhages in the lower small intestine, and similar but faded hemorrhages in the large intestine. Unfortunately, only one piece of the large and of the small intestine was saved for microscopic examination.

In the section of the small intestine every third or fourth tubule contained one to several oocysts; gametes were less numerous and schizonts scarce. The measurements indicate that elliptical coccidia are present. In the section of large intestine every tubule is invaded with coccidia chiefly in the stage of schizonts. A few oocysts are, however, present. Sections of the various viscera were negative.

Calf 49.—Red and white, female. Born Mar. 27, 1917.

Attendant called attention to this case stating that the animal had had diarrhea and blood in the discharges beginning Apr. 26, and that on Apr. 30 large amounts had been passed. On this date feces were watery and stained with blood. Tenesmus. Calf active and in good flesh. Salivation. Temperature 39.4°C. May 1. Temperature 38.4°C. Fecal blood still in evidence. Diarrhea diminished. Tenesmus increased. Slight salivation. Calf found dead in the morning of May 3.

The changes found at autopsy are briefly as follows: Mucosa of duodenum and upper jejunum sprinkled with punctiform hemorrhages aggregated into patches. Lower down there are uniformly and deeply congested patches. Moderate congestion of large intestine, except in lowest portion of rectum where it is more intense. The cephalic half of both lungs is sprinkled over with dark to bright red lobules interspersed among air-containing tissue. When the lobes are incised the central portions are found largely hepatized, the involved tissues showing dark red and lighter areas intermingled. Small whitish sclerotic foci permeating cortex of both kidneys.

Microscopic examination of fixed and hardened tissues shows foci of coccidia. These are minute and the number of parasites is not large. The prevailing forms are macrogametes, microgametocytes, and oocysts. These are of the smaller elliptical type. The infection varies greatly in intensity.

Sections from certain regions involving the entire circumference of the tube contain but a few parasites. In one section fifteen oocysts blocked one tubule. In other tubules nearby the lumen was full of debris including empty, collapsed oocysts and among them leukocytes. Only a few schizonts were detected.

In the large intestine (only one piece of tissue was available) every tubule contains fully formed masses of merozoites, which greatly predominate over the gametes in numbers.

The cause of death in this case was probably the septic pneumonia from which streptococci and staphylococci were isolated. The significance of the coccidiosis remains in doubt because only one small area of the large intestine had been preserved.

Calf 92.—Red and white, female; weight about 110 pounds. Born Apr. 24, 1917, found dead June 6.

The attendant reported that the calf had received some antidiarrhea serum soon after birth. Diarrhea, however, appeared for several weeks intermittently. June 4. Depression and loss of appetite were first noticed.

The autopsy, June 6, showed considerable postmortem changes. In the small intestine were localized congested areas. In the large intestine the mucosa had a dull, opaque appearance. Other organs appeared normal.

Microscopic examination of four different levels of the small intestine showed in each level scattering coccidia including schizonts, gametes, and oocysts. In three levels of the large intestine all epithelium still *in situ* was invaded. Thus in two levels there were chiefly the asexual stages with merozoites fully developed.

Oocysts were also present and indiscriminately scattered among the schizonts. In the third region oocysts predominated and some tubules were packed with the liberated forms. In this case the large ovoid oocyst was seen for the first time, and associated with the smaller elliptical form (Fig. 6).

Calf 102.—Holstein, female. Born Apr. 15, 1917.

May 1. Symptoms of pneumonia first appear. June 26. Emaciated; staring coat. Temperature 39.5°C. Cough, dyspnea, and slight nasal discharge. Fecal discharges normal. June 28. Killed. Calf weighs 100 to 120 pounds.

The autopsy shows extensive consolidation of lungs. Cephalic and ventral lobes symmetrically involved. Whitish, lardaceous foci in cortex of both kidneys. The digestive tract showed the following abnormalities: In the fourth stomach two erosions about 3 cm. in diameter covered with adherent thin, greenish-stained exudation. Some point-like hemorrhages in ileocecal valve and rectum. Oocysts of the smaller ellipsoidal type found in considerable numbers in feces pressed from rectum. In snippings from the fresh intestine none were detected.

In sections of the small intestine none were seen. In those of the large intestine a few gametes present in surface epithelium and several oocysts in phagocytes in intertubular tissue of mucosa. The cysts are partly collapsed. Evidently the process was nearly over.

Calf 121.—Red and white, female. Born June 25, 1917, found dead July 31.

The following details were obtained. The calf had lost most of its coat of hair at the time of death. The falling out of hair began early in July and was progressive in character. The animal had suffered from diarrhea intermittently but there was no evidence of blood-passing.

Body weighs about 70 pounds. Markedly emaciated. Skin almost free from hair; dry and harsh. Some ecchymoses and extravasations in subcutis. Gas blebs in axilla. Hair-balls embedded in large quantity of clear, viscid mucus in fourth stomach. Congestion of mucosa with scattering petechiæ. Throughout the small intestine small sharply defined hemorrhages more or less uniformly distributed. The large intestine showed nothing noteworthy. Consolidation of the ventral and the distal portion of cephalic lobe of the right lung with filling up of the air tubes of affected lobes with mucopurulent molds. Feces taken from rectum contained large numbers of oocysts of the ellipsoidal type.

Sections from six different levels of small intestine contained no coccidia. In the large intestine complete transections of the tube in three different regions revealed extensive invasion with coccidia. In one section every tubule was involved. The parasites were chiefly in the stage of asexual multiplication with a fair number of gametes and oocysts in the tubules. Many of the latter were packed with necrotic cells. In one tubule could be seen merozoites, oocysts, macro- and microgametocytes. In a second cross-section one large focus was found, the remainder of the tubules being filled with mucus-secreting cells. In the invaded area there were chiefly merozoites with a fair number of microgametocytes. The third section contained three foci of invasion in which each epithelial cell contained one to three schizonts. Gametes very rare. Decline

and death in this calf were probably due to the extensive invasion of the large intestine.

Calf 139.—Guernsey, female. Born Aug. 15, 1917.

Sept. 25. Cough first noticed. Sept. 28. Temperature 41°C. Feces soft, approaching diarrhea. Oct. 2. Removed from herd and killed because of physical signs of pneumonia.

At autopsy there was found symmetrical consolidation of the cephalic half of both lungs. The mucosa of small intestine was more or less congested throughout. Below the valve in the large intestine the patch of flask-shaped glands is deeply congested. Yellowish, soft, cheesy plugs fill the lumina of glands. Through remainder of colon scattering, point-like hemorrhages. In rectum congestion well marked. Fresh clippings of mucosa taken from valve to rectum show presence of large, ovoid oocysts in all preparations. In sections of fixed tissue, however, only a few oocysts found and these in intertubular tissue. Evidently the process had run its course.

Calf 184.—Red and white, female. Born Oct. 1, 1917.

Between Oct. 20 and 23 temperature fluctuated between 40° and 41°C. Oct. 23. Blood in feces, but coccidia not found among blood cells. Tenesmus. Dec. 5. Calf found dead. Probably *B. coli* septicemia. In feces from rectum and in snippings from mucosa of large intestine near valve a few large ovoid oocysts found. In sections from various levels of the small intestine a few gametes were detected.

Recovery from coccidiosis is illustrated by the following cases.

Calf A.—Born July 12, 1917. When 32 days old, on Aug. 13, blood appeared in the discharges associated with large numbers of oocysts. Aug. 22. Feces were again examined and one oocyst was found. Subsequent history uneventful. The calf was normal in size in Jan., 1918.

Calf B.—On Aug. 15, 1917, when the calf was 39 days old, blood appeared in feces. A considerable number of large ovoid and a few smaller elliptical oocysts were found. Jan. 17, 1918. This calf is in normal condition as regards weight.

Calf C.—Aug. 15, 1917. 32 days old. Several oocysts found. Jan. 31, 1918. Of normal size.

Calf D.—Born Aug. 11, 1917. Sept. 4. One oocyst was found in feces. This calf was not affected clinically. Jan. 31, 1918. Of normal weight.

Calf E.—Black and white, female. Born Sept. 5, 1917. Oct. 3. Diarrhea started. Oct. 5. Feces of a penetrating putrefactive odor, containing blood. Microscopic examination shows in the blood clot many ovoid as well as elliptical oocysts. Jan. 17, 1918. Calf in very good condition.

Calf F.—Yellow and white, female. Born Sept. 5, 1917. Oct. 8. Diarrhea and blood in stools noticed. Tenesmus. In the feces containing many red corpuscles are large numbers of elliptical oocysts. Jan. 17, 1918. Calf in very good condition.

In addition to the cases recorded there were seven other calves whose feces were subjected to examination with negative result. Some of these were suffering from diarrhea, others not. This fact is of interest since these calves had been in the same yard with cases of coccidiosis. It is, of course, not improbable that repeated examination might have shown coccidia in all cases.

SUMMARY.

Discharges of blood per rectum, associated with oocysts of coccidia, were observed occurring in young calves during the warmer season of the year. In a small percentage of the cases death was probably due directly to the coccidiosis. Although the disease, known as red dysentery in Switzerland, may have existed in this country for some time, there seems to have been no knowledge of its existence and no reports of it have thus far been published. The coccidia have been artificially cultivated and shown to produce four spores. Two oocysts of quite different dimensions and having minor differential characters were encountered in the same animal in several instances.

The invasion of the epithelium of the small intestine was slight. The chief seat of the parasitism was the large intestine. The lesions following the loss of epithelium were superficial hemorrhages and filling up of the denuded tubules with polymorphonuclear leukocytes.

Addendum.—During 1918, up to May 31, only one case of bloody dysentery was observed in the same herd. This occurred in a calf over 11 months old. The coccidia found in the feces were of the smaller elliptical type. The symptoms rapidly disappeared.

The clinical data in this communication were gathered by Dr. Ralph B. Little.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Blood in feces from a calf which subsequently recovered. The oocysts as contained in the fresh blood clot. × 300.

FIGS. 2 and 3. Oocysts of the smaller coccidium from a culture showing the presence of four sporoblasts. Photograph retouched.

PLATE 2.

FIG. 4. A tubule from the upper colon of Calf 34 fixed in Zenker's fluid. Almost every cell contains one to three parasites. They are indistinctly seen between cell nucleus and free margin of the epithelial cell. The accentuated small spheres within the parasites are nuclei of future merozoites (trophozoites). × 750.

FIG. 5. A tubule from the same focus of the colon of Calf 34 as that shown in FIG. 4. In this tubule the schizonts have broken up into groups of merozoites. The outline of the tubule is no longer recognizable. × 750.

PLATE 3.

FIG. 6. Large intestine of Calf 92. A large and a small oocyst in the same field. The smaller one is clearly located in a tubule, the large one in the intertubular tissue. It is not improbable that in this instance owing to the toughness of the shell it may have been dislocated in cutting or mounting the section. × 750.

FIG. 7. Large intestine of Calf 121. Note a mass of exudate adhering to the mucous membrane. The mass, made up of fibrin, blood corpuscles, leukocytes, and mucus, contains the ellipsoidal oocysts. The tubular epithelium is largely destroyed and the tubules are filled with polynuclear cells. × 90.

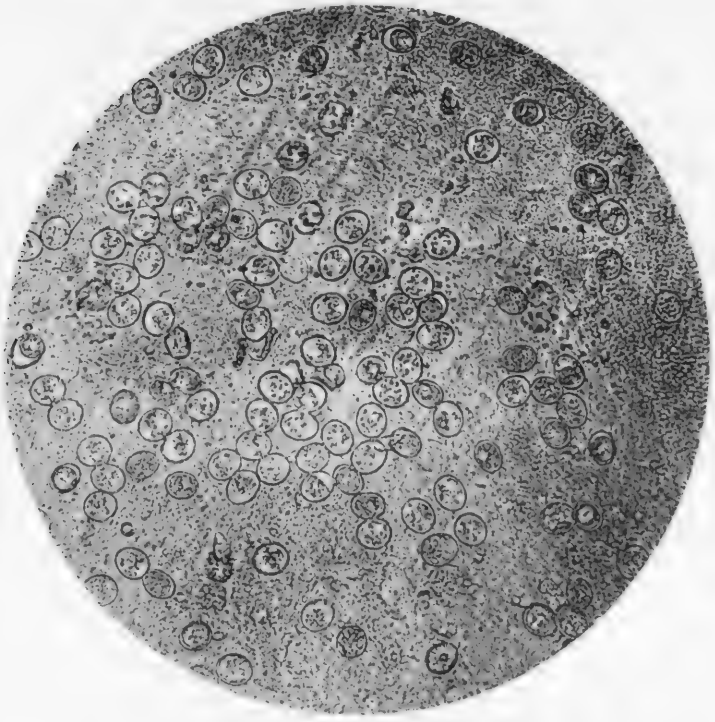

FIG. 1.

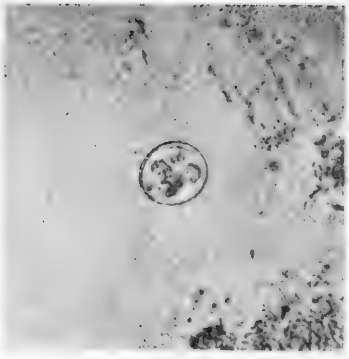

FIG. 2.

FIG. 3.

(Smith and Graybill: Coccidiosis in calves.)

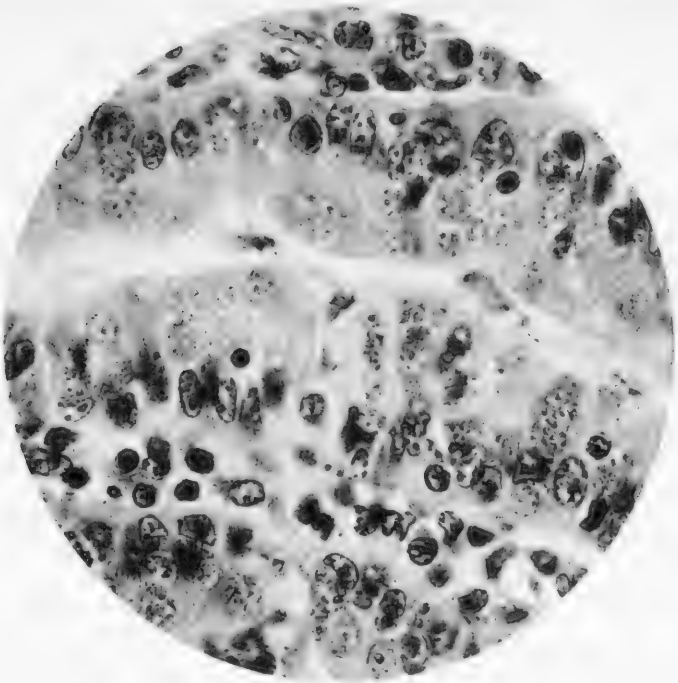

FIG. 4.

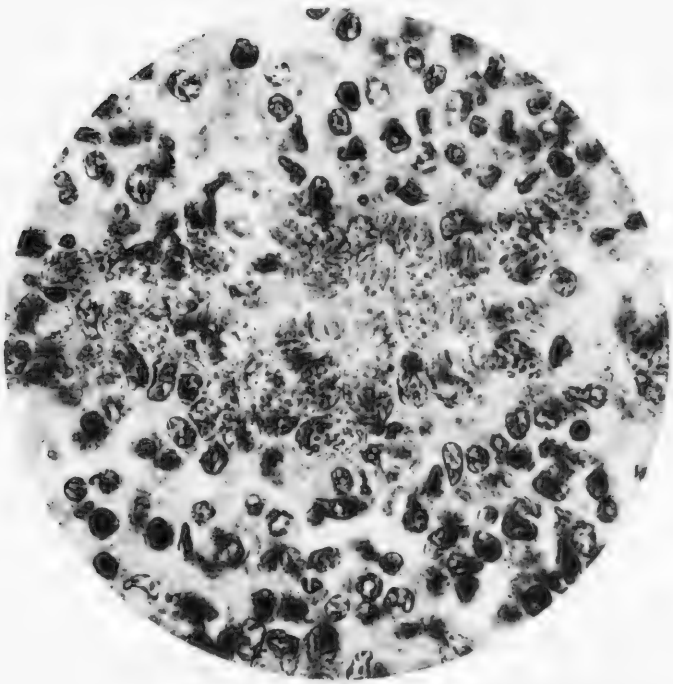

FIG. 5.

(Smith and Graybill: Coccidiosis in calves.)

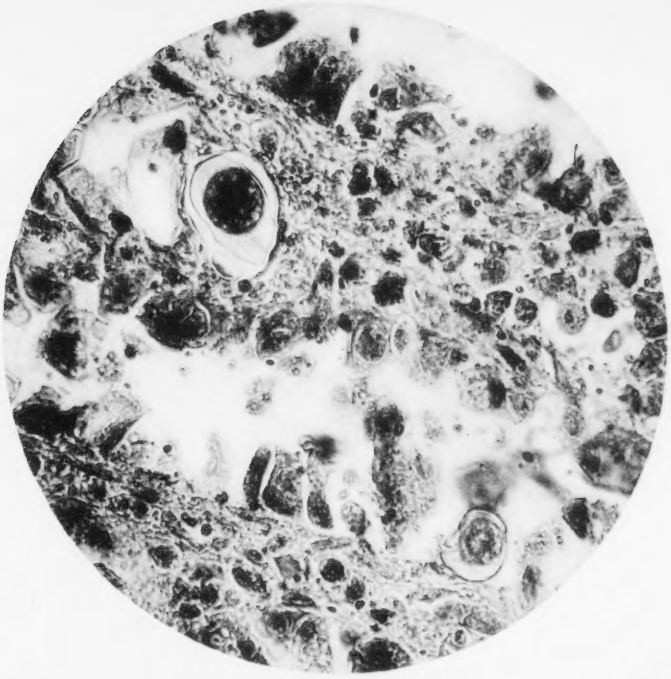

FIG. 6.

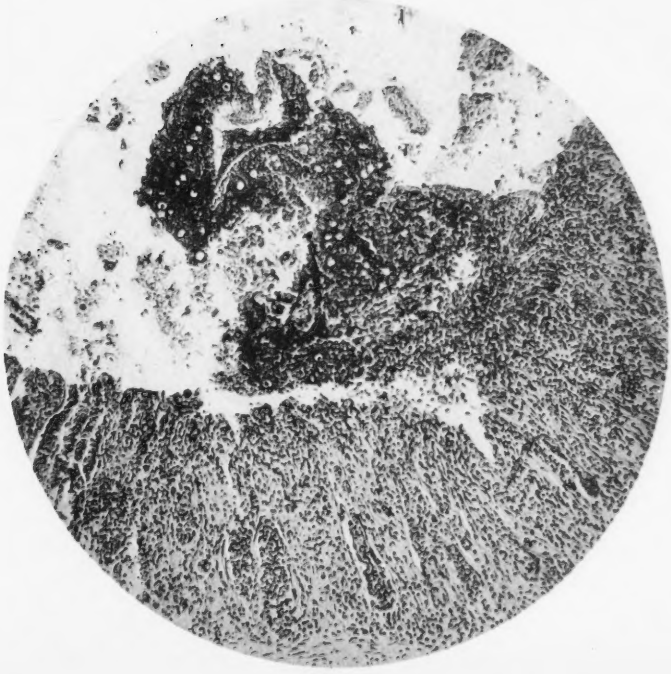

FIG. 7.

(Smith and Graybill: Coccidiosis in calves.)